The Role of E1 SUMO Activating Enzyme in the Development of *Drosophila melanogaster*

Thesis

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The role of E1 SUMO activating enzyme in the development of *Drosophila melanogaster*

PhD Thesis

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ABSTRACT

SUMOylation is an Ubiquitin-like post-translational modification thought to affect a diverse array of cellular processes, from chromosome segregation to nuclear translocation. The E1 SUMO-activating enzyme is required for the earliest steps of SUMO conjugation, and is composed of heterodimeric Aos1 and Uba2 subunits. The genes encoding both of these proteins are essential for viability in yeast, but less is known about their biological role in other organisms. Here we report a transposable element mutation in the *aos1* gene of *Drosophila* that causes complete elimination of imaginal discs (appendage primordia) in homozygous larvae, while the rest of larval tissues appear to be unaffected. We were able to rescue this growth phenotype with a genomic construct containing only the *aos1* gene, confirming that the growth defect results solely from loss of *aos1*. Mosaic analysis reveals that *aos1* mutant disc cells die through apoptosis, but when apoptosis is blocked the mutant cells exhibit cell cycle progression defects. Using FACS analysis to measure cell cycle phasing, we found that *aos1* mutant cells accumulate in G2/M, indicating a blockage in progression to mitosis. Additionally, *uba2* homozygous mutant animals also lack imaginal discs without exhibiting defects in larval tissues, indicating that SUMO E1 enzyme activity is required more in some cells than in others. Consistent with this, we show that *aos1* and *uba2* are primarily required for cell proliferation, since RNAi-mediated *aos1* and *uba2* knockdown in dividing cells of the eye discs resulted in reduction of eye size, while expression of *aos1* or *uba2* RNAi in post-mitotic cells of the eye disc had no effect. Together, these results reveal a key role for the E1 SUMO activating enzyme in cell proliferation, but they suggest that SUMO E1 activity is less required for cellular survival, differentiation, or homeostasis of non-dividing cells.

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Abbreviations

AP axis	Anterior-Posterior axis
APC	Anaphase Promoting Complex
BDGP	Berkeley Drosophila Genome Project
Cdk	Cyclin-Dependent Kinase
CNS	Central Nervous System
dsRNA	Double stranded RNA
DV axis	Dorso-Ventral axis
EMS	Ethyl Methane-Sulphonate
EtOH	Ethanol
FACS	Fluorescence Activated Cell Sorting
FLP	Flippase Recognition Target
GFP	Green Fluorescent Protein
hs	Heat Shock
JH	Juvenile hormone
MARCM	Mosaic Analysis with a Repressible Cell Marker
ME	Methanol
NEM	*N*-Ethylmaleimide
PBac	PiggyBac
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl Fluoride
PTTH	Prothoracicotropic Hormone
RFP	Red Florescent Protein
RT	Room Temperature

RT-PCR	Reverse Transcription Polymerase Chain Reaction
SAGE	Serial Analysis of Gene Expression
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SIM	SUMO Interacting Motif
SUMO	Small Ubiquitin-related Modifier
UAS	Upstream Activation Sequence
UTR	Untranslated Region

Chapter 1

Development of *Drosophila melanogaster*

Life Cycle of Drosophila

Drosophila melanogaster, under normal laboratory conditions (growth at 25° C), completes its life cycle in about 10 days (Fig 1). Initially fertilized eggs are laid by the female after mating. The process of embryogenesis lasts for 22-24 hours. The larvae hatch after the end of embryonic development and start feeding. There are three larval stages, referred to as instars. The first and the second larval instars last for 24 hour, while the third lasts for approximately 48 hours, dependent on nutritional conditions and crowding. The termination of the first and second larval instars is followed by molting to the next stage, whereas the end of the third larval stage is followed by pupa formation. During the pupal development of *Drosophila* and other holometabolous insects, the animal undergoes metamorphosis and the body is completely reformed. The adults flies eclose from their pupal case after 4-4.5 days (Ashburner et al., 2005).

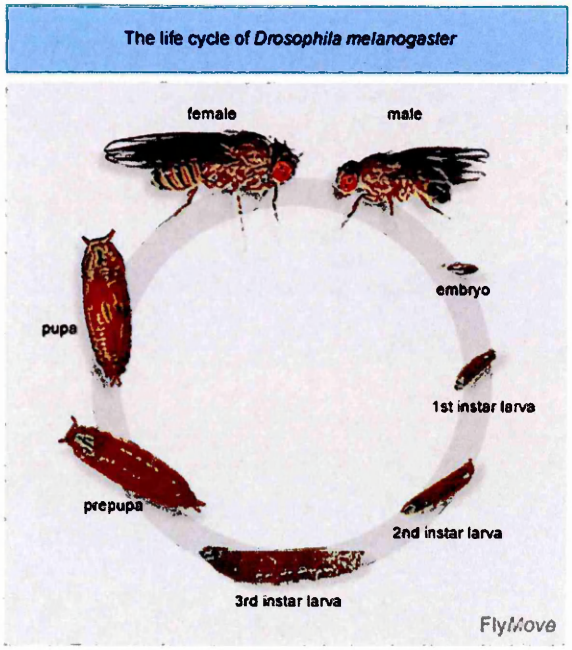

Figure 1
Life Cycle of *Drosophila melanogaster*. Upon completion of embryogenesis, animals pass through three larval instar stages. At the end of larval development, a pupa is formed. Metamorphosis takes place during pupa stage and the adult flies eclose from the pupa after ~4.5 days.

Embryogenesis and Larval development-Cell Division and Growth

Upon egg fertilization rapid divisions take place. The first 13 cycles of nuclear division that are not followed by cytokinesis and the thus the nuclei share the same cytoplasm-syncytium (Foe et al., 1993). At the 9th division cycle, most of the nuclei move to the periphery of the embryo. 5-10 nuclei in the posterior end of the embryo get separated from the rest during 10th division cycle. These nuclei will give the pole cells which are going to migrate to the gonad and become germ cells (Gilbert, 2000a; Gilbert, 2000b; Hay et al., 1988; Jaglarz and Howard, 1994). The rest of nuclei in the periphery undergo 4 more division cycles and at the 14th cycle the plasma membrane folds around them and cells begin to form (Foe and Alberts, 1983; Gilbert, 2000a; Gilbert, 2000b).

The first 13 divisions of the embryo are sustained by maternal deposition of cell cycle machinery components (Thompson, 2010). Maternally provided mRNA is also

responsible for the early polarization of the embryo along the anterior-posterior axis. After the 13th division cycle and cellularisation, the zygotic genes start to be transcribed and the cell cycle is regulated in particular patterns (Thompson, 2010). A key regulator for the switch from maternal transcription to zygotic is Smaug, an RNA-binding protein which is required for the degradation of the maternal transcripts (Benoit et al., 2009). Following mitosis 16, nervous system cells continue to divide while epidermal cells exit the cell cycle (Edgar and O'Farrell, 1990). During late embryogenesis, the larval tissues enter endoreplication cycles, while a few cells that have been determined to give rise to the adult structures continue to proliferate throughout larval development.

At the end of embryogenesis, larvae hatch and start feeding to promote their growth further. The larvae increase dramatically their size from the time they hatch until pupation. This growth is achieved through cell growth but not cell proliferation (Thompson, 2010). Larval cells undergo endoreplication cycles which consist of DNA replication that is not followed by mitosis and thus, these cells become polyploid (Edgar and Orr-Weaver, 2001). Endoreplication accelerates growth rate since increased gene expression, due to multiple gene copies, leads to increased metabolism and increased resistance upon DNA damage (Lee et al., 2009). In contrast, the cells that are destined to form the adult structures continue to divide during larval development. These cells are the imaginal disc cells (described below).

Cell cycle progression

The cell cycle in eukaryotic cells is composed of 4 different phases: S phase (DNA synthesis), M phase (Mitosis), and two Gap phases G1 and G2. The cell cycle progression is regulated by Cyclin-Dependent Kinases (Cdks) (Fig 2). In yeast there is only

one Cdk that is controlled by several cell cycle specific Cyclins (Andrews and Measday, 1998). In mouse there are several Cdks that couple with different Cyclins. However, only Cdk1 is essential for viability, while Cdks2, 4 and 6 are required in specific cell types (Santamaria et al., 2007). In *Drosophila* two Cdks are required for cell cycle progression: Cdk1 for the G2/M transition and Cdk2 for G1/S transition (Lane et al., 2000; Stern et al., 1993) .

Cdk activity is controlled by specific Cyclins that are synthesized periodically. In mammalian cells Cyclin D is required for G1/S transition, Cyclin E for the initiation of S phase, Cyclin A for the exit from the S phase and Cyclins A and B for the entry to Mitosis (Budirahardja and Gonczy, 2009). In *Drosophila* Cyclin D is not essential (Meyer et al., 2000), but Cyclin E partners with Cdk2 and is required for the S phase (Sauer et al., 1995). Also, in *Drosophila* Cyclin A has not a role in S phase like in mammals, but it partners with Cdk1 and alongside with Cyclin B-Cdk1 regulate the onset of Mitosis (Knoblich and Lehner, 1993).

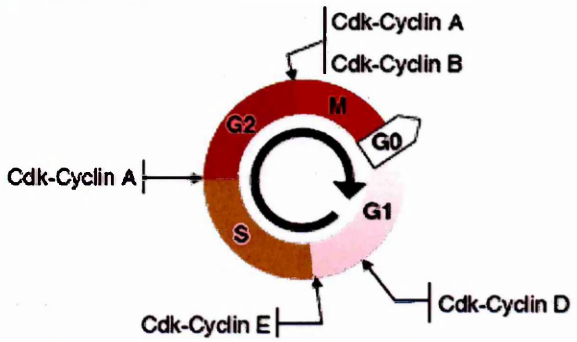

Figure 2
Cell cycle phases and the Cdk-Cyclin complexes that control the progression through them. There are four phases: DNA Syntesis (S), Mitosis (M), Gap1 (G1) and Gap2 (G2). Cdk-Cyclin D functions to promote the G1/S transition, Cdk-Cyclin E is required for the initiation of S phase, Cdk-Cyclin A for the completion of S phase (in mammal but not in *Drosophila*) and Cdk-Cyclin B together with Cdk-Cyclin A promote the entry to Mitosis. G0 is a state that cells enter when they stop diving eg. upon unfavorable environmental conditions or upon differentiation. (Budirahardja and Gonczy, 2009)

Cells that are in G1 enter S phase depending on availability of growth factors and

nutrients. Progress through G1/S is facilitated by Cyclin E upregulation (Lee and Yang, 2003). In mammals, extrinsic signals activate Cyclin D-Cdk which phosphorylates retinoblastoma protein Rb (Kato et al., 1993). Thus, the E2F transcription factor is released by the Rb inhibition and leads to transcription of Cyclin E and other proteins required for S phase (Ohtani et al., 1995). The Cyclin E-Cdk complex is additionally activated by inducing a feedback loop, as it phosphorylates further the Rb protein (Harbour et al., 1999; Hinds et al., 1992). In *Drosophila*, Cyclin D regulates growth but does not promote the G1/S transition, and developmental signals activate directly Cyclin E (Meyer et al., 2000). Exit from S phase is facilitated by the inactivation of the Cdk-Cyclin E complex after degradation of Cyclin E. SCF (Skp1/Cullin/ F-box protein) ubiquitin ligase is responsible for the targeting and subsequent proteolysis of Cyclin E (Koepp et al., 2001).

Cells enter mitosis after the activation of Cdk-Cyclin A and Cdk-Cyclin B complexes (Budirahardja and Gonczy, 2009). The abundance of Cyclin A and B is not the only requirement for Cdk activation. Wee1 kinase phosphorylates Cdk and keeps it inactive until the Cdc25 phosphatase (String in *Drosophila*) removes the inhibitory phosphate group (Edgar and O'Farrell, 1989; O'Farrell, 2001; Russell and Nurse, 1987; Stumpff et al., 2004). Degradation of mitotic Cyclins is required for completion and exit from mitosis. In *Drosophila* embryos degradation of Cyclin A takes place before the metaphase to anaphase transition, Cyclin B get degraded during the metaphase/ anaphase transition and Cyclin B3 degradation occurs during anaphase (Sigrist et al., 1995). Expression of stable forms of Cyclin A, Cyclin B and Cyclin B3 results in cell cycle arrest at metaphase, early anaphase and late anaphase respectively (Sigrist et al., 1995). Anaphase promoting complex (APC) is required for the ubiquitination and subsequent degradation of the mitotic Cyclins (Irniger, 2002). APC targets also securins and leads to their degradation, alleviating the inhibition of Separase which cleaves co-

hesion and allows the sister-chromatid disjunction (Peters, 2002).

Cell cycle progression is blocked if any of several processes are not finished correctly. Incomplete replication and DNA damage activate checkpoints during S phase or prior to mitosis entry, preventing cell cycle progression until the completion of replication or until the DNA damage is repaired (Dai and Grant, 2010). The delay in the progression of the cell cycle is achieved by the inactivation of Cdk1 and it is facilitated by kinases ATM (Ataxia Telangiectasia Mutated) and Chk1 (Checkpoint Kinase), which are known as Mei-41 and Grapes respectively in Drosophila (Fogarty et al., 1997; Hari et al., 1995). These kinases act on regulators of Cdk1, such as Cdc25 (String), whose function is blocked so that Cdk1 will be kept inactive by Wee1 (Furnari et al., 1997; Raleigh and O'Connell, 2000). Another checkpoint delays the exit from mitosis by inhibiting APC activity upon misassembled spindle. The spindle assembly checkpoint safeguards that spindle microtubules are attached correctly to the kinetochores before the initiation of anaphase (Zhou et al., 2002).

Cell cycle modulation in different developmental stages of *Drosophila*

In the beginning of embryogenesis, the 13 first division cycles are rapid and are sustained by the maternal deposition of cell cycle components. Therefore there are no Gap phases and the cell cycle is consisted by alternative S-M phases. After the cellularization at cycle 14, a G2 phase is added in order to allow the beginning of zygotic transcription (Edgar, 1995). Following the 16^{th} division cycle, the differentiating larval tissues enter endocycles with alternative S-G phases. In endocycling cells, Cdk2-Cyclin E activity oscillates, while the regulators of mitosis are downregulated so that mitosis will be prevented (Lilly and Duronio, 2005). In contrast, nervous system cells continue to divide through S-G2-M cycles even after 16^{th} division cycle.

During larval development, endoreplication is utilized by larval cells in many tissues including gut, fat body, trachea, salivary glands, and the malpighian tubules. The nervous system cells divide during larval stages using G1-S-G2-M cycles. The same division cycles are followed by the imaginal disc cells, which at pupal stage will differentiate to give rise to adult tissues. Abdominal histoblasts are also going to contribute in adult tissue formation, but remain arrested in G2 until the larval-pupal transition (Ninov et al., 2009).

Imaginal discs-growth and patterning

Imaginal discs are epithelial structures, which will form the adult appendages during pupation. The discs are formed initially from small groups of cells during embryogenesis. Late in embryogenesis they invaginate to form sac-like structures of monolayer columnar epithelium that are attached to the future larval epidermis. During larval stages the imaginal discs cells proliferate continuously, and by the end of larval development imaginal discs consist of tens of thousands of cells (Gilbert, 2000a). At metamorphosis, the imaginal discs evert out of the body and they differentiate to give rise to the adult structures (Held, 2002). There are nine pairs of imaginal discs that form the mouth parts, eyes and antennae, legs, wings and halteres (Fig 3). The head and thorax are formed by the imaginal discs, while the abdominal segments are formed by the abdominal histoblasts. Genitalia are formed from a pair of genital discs (Held, 2002).

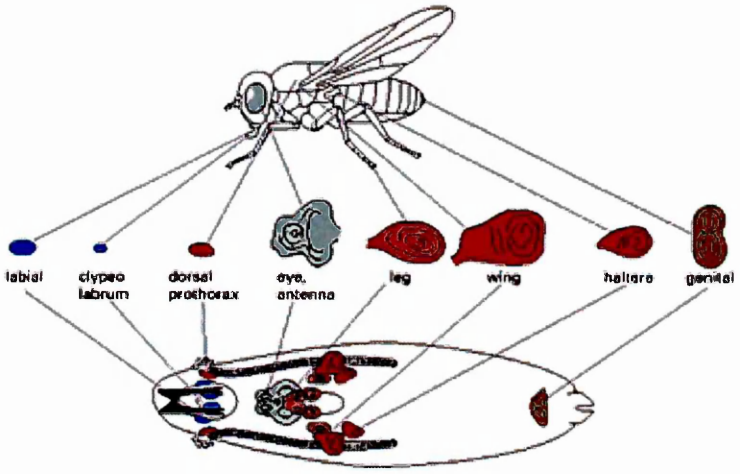

Figure 3
Imaginal discs in *Drosophila* that give rise to the adult tissues during metamorphosis. There are nine pairs of discs in the interior larval body: dorsal prothorax discs, eye antenna discs, three pairs of leg discs, wing discs, halter discs, two pair of disc that form the mouthparts. Gelinalia are formed by the genital discs.
(Alberts B, Johnson A, Lewis J, et al. 2002. Molecular Biology of the Cell. 4th edition.New York: Garland Science)

Cell division and growth in imaginal discs is controlled by developmental signaling pathways, so that cell proliferation will be coordinated in order to form an appendage of a particular shape and size. Imaginal disc cells receive positional information in which the respond by adjusting their division rate (Thompson, 2010). In the wing imaginal discs, for example, positional information is obtained through morphogens such as Dpp (Decapentaplegic), and Wg (Wingless). Dpp belongs to the TGFβ family and it is expressed in the anterior-posterior boundary of the wing disc (Posakony et al., 1990). Activation of Dpp signaling pathway induces the expression of *omb* and *sal* genes (Nellen et al., 1996). The gene *sal* is expressed by the cells that are close to the AP boundary and therefore receive a high concentration of Dpp, while *omb* is expressed in a wider domain than *sal*. Wingless belongs to the Wnt family and it is expressed in the dorso-ventral boundary of the wing imaginal disc (Diaz-Benjumea and Cohen, 1995). Similar to Dpp, depending on the proximity of cells to the DV boundary and thus the concentration of Wg they receive, the expression of different genes is in-

duced (Zecca et al., 1996). Higher to lower concentration of Wg induces the expression of *ac* (achaete), Dll (Distal-less) and *vg* (vestigial) (Diaz-Benjumea et al., 1994; Neumann and Cohen, 1996; Phillips and Whittle, 1993). Following this general logic, each cell within an imaginal disc is specified according to the AP and DV axes. This results in different combinations of gene expression which specify the appropriate positional identity for each cell. Importantly, the mechanisms through which the positional signals integrate with the control of cell growth and cell division remain poorly understood.

Pupa formation-Ecdysone

The transformation of a larva to adult is completed during pupation. At pupal stage the larval tissues are destroyed while the adult tissue progenitor cells divide and differentiate in order to form the adult structures. The process of pupa formation as well as the transition from one larval stage to the next is controlled by hormones. Ecdysone is the main hormone that regulates these transitions (Truman and Riddiford, 2002).

The prothoracicotropic hormone PTTH is a neuropeptide that is produced in the Drosophila brain and it stimulates the production of Ecdysone in the prothoracic glands (Nijhout, 1994). Ecdysone is a steroid hormone that is synthesized from cholesterol. The prothoracic gland produces a precursor of Ecdysone that is released in hemolymph and it is converted into its active form 20E in midgut, fat body and malpighian tubules (Rewitz et al., 2006). When Ecdysone is bound to its receptor, it activates genes that encode transcription factors which in turn activate genes that prepare the tissues for stage specific biological processes (Crossgrove et al., 1996; Segraves and Hogness, 1990; Thummel et al., 1990).

Ecdysone levels increase before each larval molt, during which the old cuticle is

shed and a new cuticle is formed. A higher peak of Ecdysone promotes pupa formation at the end of the 3rd instar larval stage and increased levels of Ecdysone during metamorphosis induce tissue differentiation (Gilbert, 2000a; Kozlova and Thummel, 2000; Nijhout, 1994). After the adult formation, Ecdysone levels decline.

Another important hormone that regulates the effects of Ecdysone is the Juvenile hormone JH. The JH is present during larval development and prevents metamorphosis during molting. At the larva to pupa transition the JH declines and Ecdysone in the absence of JH induces pupation (Riddiford, 1996).

Imaginal disc growth defects observed in mutation of a SUMOylation pathway enzyme

In the current study, defects caused by the disruption of the SUMOylation pathway are examined during development of *Drosophila* imaginal discs. The initial observation was that imaginal disc growth was abolished by a mutation in *aos1*, an enzyme responsible for the first step in the biochemical pathway of SUMO conjugation. Previously, no other reports have examined the general function of SUMOylation during imaginal disc development. An overview of the SUMO pathway and its functions is provided below.

Chapter 2

SUMOylation

SUMO (Small Ubiquitin-related Modifier)

SUMO belongs to the Ubiquitin-like family of proteins that includes Nedd-8 and ISG15 (Schwartz and Hochstrasser, 2003). These proteins, like Ubiquitin, can be conjugated to other proteins and alter their properties like other post-translational modifications.

The SUMO gene was discovered initially in *Saccharomyces cerevisiae* (*smt3*). It was identified as a suppressor of a temperature sensitive allele of the centromeric protein Mif2 in a genetic screen (Melchior, 2000). It was in mammals, however, that SUMO protein was found to be covalently attached to another protein. The first pro-

tein that SUMO was found to be conjugated to was the GTPase-activating protein, RanGAP1 (Mahajan et al., 1997; Matunis et al., 1996). All eukaryotic organisms express SUMO. There are SUMO genes in protozoa, plants, fungi and metazoa but they are absent in bacteria and archaea (Meulmeester and Melchior, 2008). Yeast, insects and worms have a single SUMO gene, while higher eukaryotes have several. Plants express up to eight versions of SUMO (Hay, 2005). Mammals have at least 3 SUMO genes: SUMO1, SUMO2 and SUMO3 (Melchior, 2000). In the human genome there is a fourth SUMO gene, but is it unclear if SUMO4 protein can be conjugated to other proteins. SUMO1, 2 and 3 are expressed ubiquitously in all tissues and developmental stages, while SUMO4 seems to be expressed only in kidney and spleen (Johnson, 2004; Meulmeester and Melchior, 2008).

SUMO proteins are only 18% identical with Ubiquitin in their primary sequence, but their folded structure resembles that of Ubiquitin. The charge distribution in their surface however is very different from Ubiquitin (Bayer et al., 1998). SUMO proteins are about 11kD in molecular weight but they appear at a higher size in SDS-PAGE. When they are attached to a substrate they add about 20kD in the molecular weight of the protein (Johnson, 2004). SUMOs are approximately 20 amino acids longer than Ubiquitin. SUMO in *Drosophila* is 90 amino acids. These extra residues are found in the unstructured N-terminal domain of the SUMO protein (Bayer et al., 1998).

SUMO conjugation pathway

The process of SUMO addition to substrates is called SUMOylation. All SUMO proteins are encoded in the genes as precursor molecules that have an extra C-terminal peptide which is removed by cleavage. This maturation step is necessary so that the C-terminal Glycine-Glycine motif can be exposed and used for the conjugation. The di-

Glycine motif is found in most Ubiquitin-like proteins (Geiss-Friedlander and Melchior, 2007). The processing of SUMO is carried out by specific proteases which are able also to remove SUMO from substrates.

The biochemical pathway of SUMO conjugation resembles that of Ubiquitin and it is evolutionally conserved (Fig 4). The SUMO conjugation process starts with the activation of SUMO by the E1 SUMO activating complex which is an Aos1/Uba2 heterodimer (Johnson et al., 1997). SUMO is activated by the formation of a thioester bond between the catalytic Cystein of the Uba2 subunit and the C-terminal Glycine of SUMO. ATP hydrolysis is required for this step. Secondly, the activated SUMO is transferred to the catalytic Cystein of the E2 SUMO conjugating enzyme, Ubc9. Finally the SUMO is transferred to the ε-amino group of a Lysine side chain of the target protein with the assistance of E3 SUMO protein ligases. Between the Lysine of the substrate and the SUMO Glycine an isopeptide bond is formed (Bossis and Melchior, 2006).

Many target proteins of SUMOylation contain a specific SUMO consensus motif ψKxE. ψ is a large hydrophobic amino acid mainly Isoleucin, Leucin or Valine, K is the Lysine residue where the SUMO gets attached, x is any residue and E is the Glutamic acid (Rodriguez et al., 2001). Ubc9 recognizes and binds directly this motif. However, not all protein substrates have this motif and many proteins are modified at sites other than ψKxE.

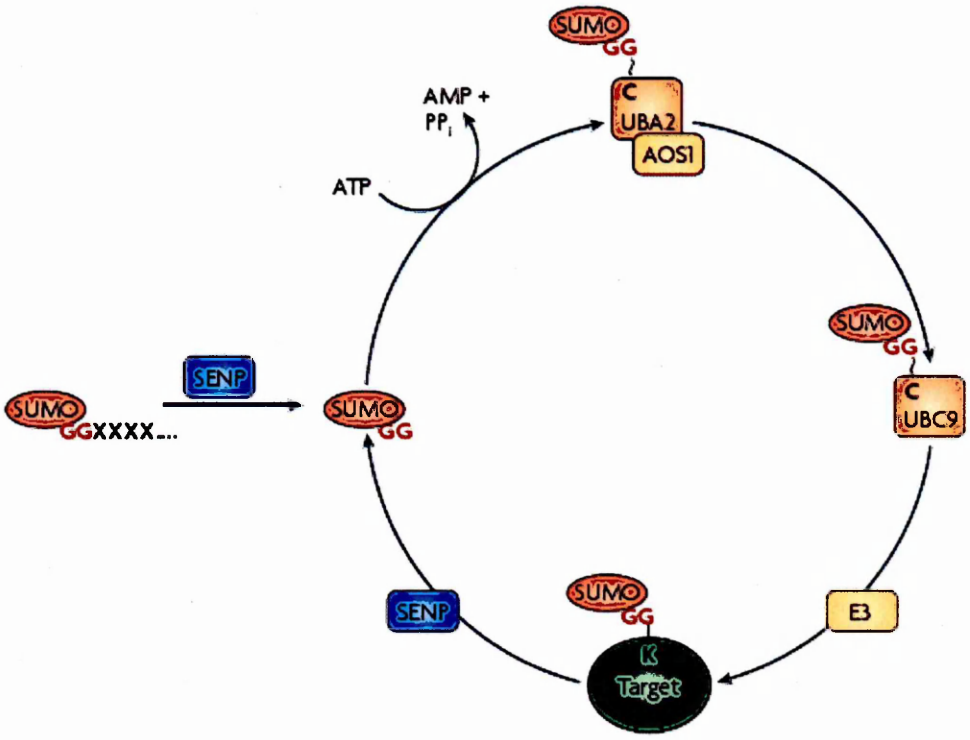

Figure 4
SUMO conjugation pathway. Initially SUMO precursor molecules are processed by SENP proteases so that the di-Glycine motif in their C-terminal will be exposed. The mature SUMOs are activated by the Aos1/Uba2 heterodimeric E1 SUMO activating complex. A thioester bond is formed between the C-terminal Glycine of SUMO and the catalytic Cystein of Uba2. The reaction requires ATP hydrolysis. Subsequently, SUMO is transferred to the Ubc9 E2 SUMO conjugation enzyme. Finally Ubc9 transfers SUMO to a Lysine residue of target proteins assisted by E3 SUMO ligases. SENPs recognize and de-conjugate SUMO from substrates, so SUMO is released and becomes available for a new conjugation cycle (Geiss-Friedlander and Melchior, 2007).

SUMOylation is a reversible protein modification and there are proteases that remove SUMO from substrates (Mukhopadhyay and Dasso, 2007). Deconjugation releases the SUMO protein which is available to start another conjugation cycle. SUMO attachment to proteins is very unstable and cleaving enzymes deSUMOylate rapidly all conjugates upon cell lysis. Therefore, with most experimental treatments, the SUMO conjugation is lost. It is required to use denaturing conditions or inhibitors of the cleaving enzymes in order to recover SUMO conjugated protein (Meulmeester and Melchior, 2008). In addition, only a small percentage of the substrate is SUMOylated at a steady state. This percentage can be even less than 1% in many cases (Johnson,

2004). These facts caused a delay in the discovery of SUMO conjugation which was only found in '96-'97 in the RanGAP1 protein, where SUMO modification is unusually stable (Mahajan et al., 1997; Matunis et al., 1996).

SUMOylation is essential in most organisms: budding yeast, nematodes, fruit flies, mice, and plants (*Arabidospis thaliana*). In multicellular organisms SUMOylation is thought to take place in all tissues and developmental stages (Geiss-Friedlander and Melchior, 2007). It affects many biological processes and it is required for cell viability (Hay, 2005). The only exception known today is fission yeast. In fission yeast (*S.pombe*), upon disruption of the single SUMO gene, the cells are sick but viable (Tanaka et al., 1999).

SUMO1 and SUMO2/3 isoforms and SUMO chains

SUMO proteins in metazoans can be categorized into two subfamilies: SUMO1 and SUMO2/3. The mammalian SUMO2 and SUMO3 proteins are about 95% identical in their primary amino acid sequence with each other, while they are only 50% identical with SUMO1 (Johnson, 2004). *Xenopus laevis* and zebrafish have both SUMO1 and SUMO2/3 (Melchior, 2000). *C.elegans* has a single SUMO gene that encodes a SUMO protein that falls into SUMO1 family (Choudhury and Li, 1997). *Drosophila melanogaster* has a single SUMO that falls into SUMO2/3 category (Huang et al., 1998) and mammals have all SUMO1, SUMO2 and SUMO3 proteins. The SUMOs in fungi and plants form distinct groups from the SUMO1 and SUMO2/3 categories (Melchior, 2000).

All SUMO isoforms are conjugated by the same E1 and E2 enzymes. SUMO1 seems to have distinct function from SUMO2/3, while SUMO2 and SUMO3 are assumed to have no functional differences. It is not known yet whether each individual SUMO is

essential in organism viability. It seems that SUMO1 deficiency cannot be compensated by SUMO2/3 in mice as it is reported that SUMO1 disruption results in embryonic lethality (Alkuraya et al., 2006). However, this result was not reproduced in later study (Zhang et al., 2008).

In mammals, it has been shown that different substrate proteins are preferentially modified with different SUMO isoforms. An example is the RanGAP1 which is mainly modified by SUMO1 but is not strongly modified by SUMO2/3 (Johnson, 2004), while Topoisomerase II is predominantly modified by SUMO2/3 (Gill, 2004). Some target proteins are modified equally by SUMO1 and SUMO2/3. The conjugation of the different SUMO isoforms to the substrates is probably regulated by the E3 ligases, although the selection mechanism is not known yet. SUMO1 and SUMO2/3 are also differentially affected by the SUMO proteases (Meulmeester and Melchior, 2008).

Another difference between SUMO1 and SUMO2/3 is that at any given time the vast majority of SUMO1 in the cell is conjugated to other proteins whereas there is a large pool of free SUMO2/3 (Johnson, 2004). The conjugation of SUMO2/3 is highly increased in response to various stresses. This could reflect a different regulation of SUMO2/3 conjugation or just the consequence of having a large reservoir of free SUMO2/3 available in the cell while free SUMO1 is limited (Bossis and Melchior, 2006). Therefore, one function of SUMO2/3 may be to provide a large pool of free SUMO for response to stress.

In addition, there is a difference in the capacity for chain formation between SUMO1 and SUMO2/3. SUMO2 and SUMO3 have a SUMO consensus motif ψKxE in their unstructured N-terminal region. This consensus can serve as an attachment site for SUMOylation, thus allowing SUMO2/3 to form poly-SUMO chains (Ulrich, 2008). The target Lysine of the consensus motif in SUMO2/3 is K11. Yeast SUMO (Smt3) has three such motifs around K11, K15 and K19 and it can also form chains.

This SUMO acceptor site is absent from SUMO1. *In vitro*, SUMO1 can form chains through non-consensus Lysines, but less efficiently (Ulrich, 2008). SUMO1 has been found conjugated to SUMO2 or SUMO3 in cells, serving probably as a terminator of poly-SUMO chains.

In vivo most Lysine residues that are modified have a single SUMO molecule attached to them and often proteins are multiply modified by attachment of mono-SUMO at different sites. To date, poly-SUMO chains have been observed in cells only on a few proteins: the Histone deacetylase HDAC4 in mammalian cells, the transcriptional regulator HIF-1α and the PCNA in yeast which was found to carry an oligomeric Smt3 chain (Matic et al., 2008; Tatham et al., 2001; Windecker and Ulrich, 2008).

The function of SUMO chains is not clear. In yeast, chain formation is not essential for viability and inhibition of Smt3 polymerization through mutation of the acceptor Lysines has no effect on vegetative growth. However, SUMO chain formation is required for efficient sporulation, which suggests a role of poly-SUMO chains in meiotic cell division (Ulrich, 2008).

The E1 SUMO activating enzyme (Aos1/Uba2)

The E1 SUMO activating enzyme catalyzes the first step of SUMO conjugation. It is a heterodimer consisted of Aos1 and Uba2 proteins and both subunits are conserved from yeast to human. In most organisms there is only one E1 SUMO activating enzyme that is required for the conjugation of all SUMO isoforms on substrates. Only *Arabidopsis* has been found to contain two Aos1 (SAE1) genes. Each of these proteins forms a pair with the single Uba2 (SAE2) protein (Johnson, 2004).

The Aos1/Uba2 enzyme resembles the E1 ubiquitin activating enzyme, although the

latter is monomeric. In *S. cerevisiae* Aos1 is 29% identical with the N-terminal region of Ubiquitin E1 (Uba1), while Uba2 is 28% identical to the C-terminal region of Uba1 (Melchior, 2000). The human E1 SUMO activating enzyme crystallization revealed that the Aos1 and Uba2 dimerization occurs through the two conserved adenylation domains, one in each subunit (Fig 5). The adenylation domain protein sequence in Uba2 surrounds the protein sequence of the catalytic Cystein domain. Another domain in the Uba2, the Ubl domain that is found in the C-terminal of all E1 enzymes, is responsible for recruiting the E2 enzyme for the transfer of SUMO to E2 (Lois and Lima, 2005).

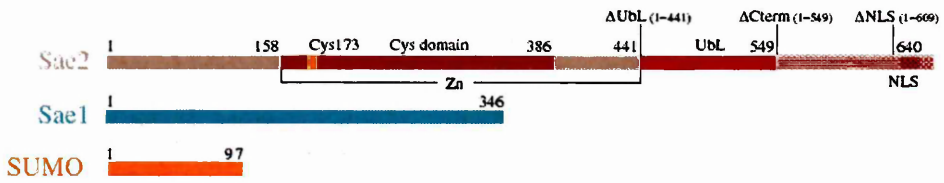

Figure 5
The domains of human Aos1/Sae1 and Uba2/Sae1 E1 subunits. Uba2 contains the catalytic Cystein (yellow) that forms thioester bond with SUMO. The Uba2 adenylation domain surrounds the sequence of the catalytic Cys domain (pink) and it interacts with the Aos1 which also contains an adenylation domain. The UbL domain in Uba2 (red) is required for recruitment of the E2 enzyme so that SUMO can be transferred to E2. (Lois and Lima, 2005)

Despite the heterodimeric form of the SUMO E1 activating enzyme, the Aos1 and Uba2 subunits do not seem to be regulated or function separately. Uba2 alone is not sufficient for the formation of thioester bond and a second protein is required (Johnson et al., 1997). In the same study it was shown that *in vitro* Aos1 and Uba2 can form an active E1 SUMO activating enzyme. In addition all cellular Uba2 and Aos1 are found as heterodimers (Johnson, 2004). Aos1 and Uba2 are likely to be expressed ubiquitously in mammals, since cDNAs for both have been found in many different embryonic and adult tissues and also in culture cells. In *S.cerevisiae* both *aos1* and *uba2*

genes are essential for viability (Melchior, 2000). In *Drosophila*, nothing is known about the biological requirements for *aos1* and *uba2* specifically, or the SUMO E1 activity generally.

The E2 SUMO conjugation enzyme (Ubc9)

After activation of SUMO by the E1 activating enzyme, SUMO is transferred to the E2 conjugation enzyme. A thioester is formed between SUMO and Ubc9, which serves as intermediate for the transfer of SUMO to the substrate. Ubc9 is the only E2 SUMO conjugation enzyme in all organisms while there are more than 20 E2 enzymes for Ubiquitination (Geiss-Friedlander and Melchior, 2007; Gill, 2004). Ubc9 is conserved from yeast to human. Like the genes that encode for SUMO, Aos1, and Uba2, the Ubc9 gene is essential in yeast and in all organisms tested thus far, except in *S.pombe* (Johnson, 2004).

The SUMO consensus motif ψKxE in substrate proteins is recognized and bound directly by Ubc9 (Bernier-Villamor et al., 2002). This is the reason that Ubc9 and Aos1/Uba2 enzymes are sufficient for SUMOylation of substrates *in vitro*. However, except RanGAP1, the *in vitro* SUMOyation of other substrates with the presence of only Ubc9 and Aos1/Uba2 is not very efficient, suggesting that E3 ligases are required to accelerate the process of SUMO transfer to substrate (Hay, 2005). *In vivo*, E3 ligases are probably regulating substrate selection, especially for substrate proteins that lack the SUMO consensus motif.

E3 SUMO Ligases

The E3 SUMO ligases facilitate the transfer of SUMO from the E2 conjugation enzyme to the target Lysine residue of the substrate. The main category of SUMO E3 li-

gases that have been found so far consist of proteins that are characterized by the presence of SP-RING motif. This motif is similar to the RING finger in E3 Ubiquitin ligases and it is essential for the E3 SUMO ligase function (Kahyo et al., 2001; Takahashi et al., 2001). The SP-RING E3 SUMO ligases function as adaptor proteins for Ubc9-SUMO thioester and substrate, so they facilitate SUMO transfer by enhancing interaction between E2 enzyme and target proteins, or by positioning them in a favorable orientation for the transfer (Geiss-Friedlander and Melchior, 2007).

The SP-RING E3 SUMO ligases include the PIAS family members (Protein Inhibitor of Activated STAT transcription factors). The PIAS family has two members in *S.cerevisiae* (Siz1 and Siz2) which are required for most SUMO conjugation in yeast, as shown in *siz1, siz2* double mutants (Johnson and Gupta, 2001). Siz1 is required for SUMOylation of PCNA and septins (Johnson and Gupta, 2001; Stelter and Ulrich, 2003). Five members of PIAS family have been found in mammals: PIAS1, PIASy, PIAS3, PIASxα and PIASxβ (Geiss-Friedlander and Melchior, 2007). PIAS1 and PIAS3 proteins are expressed in all cell types, while PIASx and PIASy seem to be expressed mainly in testis. PIAS proteins are found in the nucleoplasm and in PML nuclear bodies (Johnson, 2004).

Other SP-RING E3 SUMO ligases are the MMS21 in yeast (NSE2), which is a subunit of the multiprotein complex SMC5-SMC6 that is required for vegetative growth and for DNA repair (Andrews et al., 2005; Potts and Yu, 2005; Zhao and Blobel, 2005), and the yeast protein Zip3 which is meiosis specific (Cheng et al., 2006).

Another type of E3 SUMO ligase is the nuclear pore protein RanBP2 in vertebrates. It is located at the cytoplasmic fibrils of the nuclear pore complex. It has a catalytic domain that interacts with Ubc9 and SUMO but not with substrates, which suggests that RanBP2 does not act as an adaptor between E2 and target proteins like the SP RING E3 ligases. It is likely that RanBP2 accelerates the reaction by positioning

Ubc9-SUMO thioester in such a way that the capacity of SUMO transfer to substrate is increased (Reverter and Lima, 2005). *In vivo* there are no known targets of RanBP2, but *in vitro* it enhances the SUMOylation of Histone deacetylase HDAC4, Sp100 and PML (Geiss-Friedlander and Melchior, 2007; Kirsh et al., 2002; Pichler et al., 2002; Tatham et al., 2005).

E3 SUMO ligase activity has also been found in the Pc2 protein of the polycomb group. Polycomb group protein complexes have a Histone methylation activity and they participate in gene silencing. Pc2 recruits in Polycomb group complex the CtBP transcriptional corepressor and it enhances its SUMOylation in vivo and in vitro (Kagey et al., 2005; Kagey et al., 2003). It is not very clear how Pc2 facilitates the SUMO transfer, but it is likely that the C-terminal of the protein recruits Ubc9 and CtBP in Polycomb group subnuclear domains and the N-terminal positions the Ubc9 for efficient transfer of SUMO to CtBP (Hay, 2005). HDAC4 has been suggested to have an E3 SUMO ligase activity as well. HDAC4 is a SUMOylation target but it also enhances SUMOylation of other proteins (MEF-2, LXRβ, HIC1). In addition HDAC4 binds Ubc9 and the deacetylase activity of HDAC4 is not always required for the enhancement of SUMOylation of other proteins, therefore it is possible that HDAC4 functions as E3 ligase too (Geiss-Friedlander and Melchior, 2007).

SUMO Deconjugation Enzymes (Ulp/SENPs)

The processing of newly synthesized SUMO to its mature form and the removal of conjugated SUMO from substrates is carried out by specific SUMO proteases. These proteins were initially identified in yeast (Ulp proteins). In *S.cerevisiae* there are 2 such proteases Ulp1 and Ulp2 (Li and Hochstrasser, 1999; Li and Hochstrasser, 2000). Based on sequence similarity with Ulp1, 8 protein homologues were found in humans

(SENPs 1-8). Later studies revealed that only 6 of them are true SUMO specific proteases, as SENP3 and SENP4 were discovered to be the same protein and SENP8 is a Nedd-8 specific protease and not a SUMO protease (Mukhopadhyay and Dasso, 2007).

SUMO proteases are characterized by a catalytic isopeptidase domain of about 200 amino acids that contains the catalytic Cystein-Histidine-Asparagine triad (Cystein proteases). This catalytic domain is typically located in the C-terminal region of the proteases, while the N-terminal region is important for the intracellular localization of each protease (Melchior et al., 2003). In addition to their isopeptidase catalytic domain, some of the SUMO proteases have also a hydrolase catalytic domain in the C-terminus which is required for the maturation of SUMO precursors (Melchior et al., 2003). The SUMO proteases can be divided in two evolutionary subgroups: one that contains Ulp1, SENP1, SENP2, SENP3 and SENP5 and the other that contains Ulp2, SENP6 and SENP7 (Mukhopadhyay and Dasso, 2007).

In yeast, Ulp1 is essential for viability. Overexpression of mature SUMO in the ulp1 mutant cells rescues weakly the phenotype, which shows that Ulp1 is required for processing of SUMO but its function in SUMO deconjugation is also important (Li and Hochstrasser, 1999). Ulp2 is not essential for vegetative growth but it is required for meiotic division, chromosome segregation and temperature sensitive growth (Li and Hochstrasser, 2000; Strunnikov et al., 2001). Ulp2 seems to have only a minor role in SUMO processing but it has a role in SUMO deconjugation and especially in deconjugation of SUMO chains. Deletion of Ulp2 results in accumulation of high molecular weight conjugates, which are eliminated in the presence of SUMO that has mutated acceptor sites for chain formation (Bylebyl et al., 2003). Ulp1 is localized in the nuclear pores, while Ulp2 is found in the nucleoplasm.

Like Ulp1, the proteases SENP1 and SENP2 are active in SUMO processing.

SENP1 catalyzes more efficiently SUMO1 processing and less efficiently SUMO2 processing, while SENP2 is more efficient in the catalysis of SUMO2 maturation than in SUMO1 (Reverter and Lima, 2004; Shen et al., 2006; Xu and Au, 2005). SUMO3 can be processed efficiently by SENP5, but SUMO1 cannot (Di Bacco et al., 2006; Gong and Yeh, 2006). This substrate preference seems to be attributed to the different C-terminal extension sequence of the immature SUMO isoforms beyond the diGly motif (Mukhopadhyay and Dasso, 2007).

The mature forms of SUMO2/3 are almost identical so proteases cannot distinguish them in deconjugation, while SUMO1 is discriminated during deconjugation. Preferential deconjugation by SENPs can occur also between different substrates. For example SENP1 can distinguish SUMO1-RanGAP1 from SUMO1-Sp100 (Shen et al., 2006). Probably this is due to different structures of target proteins that cannot be bound by certain proteases. Similar to Ulp1, SENP1 and SENP2 are associated to the nuclear pores. SENP3 and SENP5 are localized in the nucleoli. SENP6 and SENP7 are localized in the nucleoplasm and SENP6 is not involved in SUMO processing, and but it can deconjugate SUMO2/3 chains like Ulp2 (Mukhopadhyay and Dasso, 2007).

SUMOylation and downstream effectors (SIM proteins)

SUMOylation changes the molecular surface of the substrate proteins. Therefore its presence on a target protein can either inhibit protein-protein interaction by masking a binding site or create a new protein-protein interaction. In the second case, it has been found that some proteins contain SUMO Interacting Motifs (Liu et al.), domains that allows proteins to bind SUMO non-covalently and thus to interact with SUMOylated proteins. The SIM motif consists of a short hydrophobic sequence flanked by acidic amino acid residues (Geiss-Friedlander and Melchior, 2007). The hydrophobic part

usually contains a sequence of four amino acids V/I/L - X - V/I/L - V/I/L (Geoffroy and Hay, 2009).

SIM domains have been found in the SUMOylation enzymes such as Uba2 and the PIAS E3 ligases, and it seems to contribute in their enzymatic function (Geiss-Friedlander and Melchior, 2007). The SIM motif has also been found in some E3 ubiquitin ligases that recognize SUMOylated proteins and promote their polyubiquitination and subsequent degradation by the proteasome. Such proteins are the Slx5-Slx8 heterodimer in *S.cerevisiae* and the *Rfp1-Slx8* and *Rfp2-Slx8* heterodimers in *S.pombe*. The Slx8 subunit appears to be responsible for the RING-dependent ubiquitin ligase activity while the other subunit recognizes SUMO via the SIM domains that it carries (Ulrich, 2008). In mammals, RNF4 is the homolog of these yeast proteins. RNF4 is a RING finger E3 ubiquitin ligase that has in its N-terminal region four potential SIM domains and it has a strong affinity of SUMO chains (Geoffroy and Hay, 2009).

Regulation of SUMOylation

Many proteins undergo SUMO conjugation and deconjugation cycles, depending on internal or external stimuli. Given the limited number of enzymes in the SUMO conjugation pathway, a critical question is how SUMOylation is regulated. Some mechanisms that regulate SUMOylation at the substrate level or at the cell level are reported below.

Changes in the localization of the substrate protein or the E3 SUMO ligase /SUMO protease that recognize the substrate can influence the SUMOylation at the substrate level. An example is the change in the localization of Siz1 (E3 ligase) in *S.serevisiae*. Siz1 is nuclear during interphase, but during mitosis it translocates in the bud neck where it promotes SUMOylation of the cytoskeletal proteins Septins (Johnson and

Gupta, 2001).

Also, it has been showed that Ubc9 from yeast and mammals is SUMOylated in vitro and in vivo. SUMOylation of Ubc9 affects the substrate specificity and enhances (Sp100) or decreases (RanGAP1) SUMOylation of certain targets (Knipscheer et al., 2008). The alteration of Ubc9 target specificity is due to the expansion of the Ubc9 binding surface upon SUMOylation. The SUMO protein that is attached to Ubc9 is recognized by the SIM domain of the substrate, when the SIM is located at an appropriate site for the interaction with SUMO. This binding between the SIM domain of the substrate and the SUMOylated Ubc9 enhances the interaction between enzyme and target, resulting in more efficient SUMO transfer (Knipscheer et al., 2008).

Another way to achieve SUMOylation target specificity is the interaction with other post-translational modifications. Phosphorylation of some targets enhances their SUMOylation (Geiss-Friedlander and Melchior, 2007). A phosphorylation-dependent SUMOylation motif has also been found, which is the SUMO consensus motif followed by a phosphorylated Serine and a Proline (ψKxExxS^{p}P). This motif has been found in various proteins (HSF1, SNIP1, MEF2). The phosphate group probably enhances the interaction between the substrate and the Ubc9. Phosphorylation can have a negative effect in SUMOylation of some targets as well (IκBα, p53, c-Jun, c-Fos) (Geiss-Friedlander and Melchior, 2007). It is not known yet how phosphorylation regulates negatively SUMOylation but it could be due to the masking of the SUMO acceptor site in these targets or due to a change in their subcellular localization.

Other post-translational modifications that interfere with SUMOylation are Ubiquitination, Acetylation and Methylation, as they all modify Lysine residues and they can compete for the same Lysine in a substrate. For example Acetylation and SUMOylation modify the same Lysine residue in Sp3 (Sapetschnig et al., 2002), while Ubiquitination and SUMOylation modify the same Lysine in IκBα and PCNA (Desterro et al.,

1998; Hoege et al., 2002). Depending on the target, the different post translational modifications can prevent each other (IκBα) or they are regulated independently upon different signals in order to serve different functions of the target protein (PCNA).

Regulatory mechanisms that target the enzymes of the SUMOylation pathway can affect global SUMOylation. Various stresses like heat shock, osmotic stress and oxidative stress cause a dramatic change of SUMO conjugation. Heat shock and osmotic stress increase global SUMOylation (Kurepa et al., 2003; Saitoh and Hinchey, 2000), while oxidative stress results in loss of global SUMOylation (Bossis and Melchior, 2006). The decrease in SUMOylation is caused by the inhibition of SUMO conjugation enzymes by the induction of a disulfide bridge between the catalytic Cystein of Uba2 and the catalytic Cystein in Ubc9 (Bossis and Melchior, 2006). The mechanism of increase in global SUMOylation is not known yet.

Finally, it has been shown that viral proteins can cause alterations in global SUMOylation. Gam1 protein from the CELO adenovirus causes a dramatic decrease in SUMOylation when it is expressed in cells. The decrease in SUMOylation is caused by the elimination of the E1 SUMO activating enzyme and E2 SUMO conjugation enzyme. Gam1 interacts with Aos1 subunit and it brings it in proximity with an E3 ubiquitin ligase which results in ubiquitination of the E1 enzyme and subsequently its degradation (Chiocca, 2007). Gam1 induces the degradation of Ubc9 as well, but the mechanism is unknown.

Functions of SUMOylation

SUMOylation is required for several cellular processes, and protein targets of SU-

MOylation are found to participate in many different biochemical pathways. Some functions of SUMOylation are described bellow.

Cell cycle

SUMOylation is involved in multiple stages of the cell cycle. In budding yeast, defects in SUMO conjugation by depletion of Ubc9 (Seufert et al., 1995) or in SUMO deconjugation by depletion of the Ulp1 protease (Li and Hochstrasser, 1999) result in cell cycle arrest in G2/M. This demonstrates the importance of SUMOylation in cell cycle progression.

SUMOylation is also required for chromosome cohesion. Cohesion of the sister chromatids ensures that the chromosomes are paired and aligned properly and that their dissociation will occur at the right time of mitosis. Pds5 in *S.cerevisiae* associates with the cohesion protein complex and is required for the maintenance of chromosome cohesion (Watts, 2007). Pds5 is SUMOylated *in vivo* in a cell cycle dependent fashion. Its SUMOylation begins before S phase and it is maximal at anaphase (Stead et al., 2003). Therefore, it seems that SUMOylation of Pds5 enables the release of cohesins in anaphase so chomosomes can segregate.

Another target of SUMOylation that is involved in chromosome cohesion is the Topoisomerase II. Topo II is SUMOylated both in yeast and in vertebrates and its SUMOylation during mitosis is required for the chromosome segregation (Azuma et al., 2003). Also the centromeric protein MIF2 (CENP-C in vertebrates), which is essential for chromosome segregation, interacts genetically with SUMO and that led to the discovery of SUMO gene (Melchior, 2000).

In addition, SUMOylation is required for APC (Anaphase Promoting Complex) mediated proteolysis of mitotic Cyclins and securin Pds1 (Dieckhoff et al., 2004; Seufert et al., 1995). Degradation of mitotic Cyclins is required for mitotic exit, while degra-

dation of Securin allows the activation of Separase. Separase in turn acts on cohesins and releases the two sister chromatid cohesion so that the chromosome segregation can take place.

Gene expression

SUMOylation can affect gene expression by regulating the assembly and activity of transcription factors or the recruitment of chromatin modifying enzymes. SUMO can also be a part of the Histone modification code as Histone H4 is shown to be SUMOylated (Hay, 2005).

SUMOylation can have a positive or a negative influence in the transcription. SUMOylation of the heat shock transcription factors HSF1, HSF2 and the p53 protein increases their transcriptional activity (Goodson et al., 2001; Gostissa et al., 1999; Hong et al., 2001). In most cases, however, SUMOylation of transcription factors results in repression of transcription. Mutation of the SUMO acceptor site in Elk-1, STAT-1, Sp-3 has been shown to increase the transcription from the responsible promoters (Johnson, 2004). For some transcription factors SUMOylation can negatively affect their synergistic binding in the multiple binding sites of promoters, like in case of androgen receptor and the glycocorticoid receptor (Holmstrom et al., 2003). In other transcriptional factors like Smad4, SUMOylation can activate or repress transcriptional activity depending on the target promoter (Hay, 2005).

Trancriptional repression upon SUMOylation can be achieved by the recruitment of co-repressors that modify chromatin structure. For instance, upon SUMOylation, p300 and Elk-1 recruit in the promoter Histone deacetylase HDAC enzymes that induce changes in chromatin and result in inhibition of transcription (Girdwood et al., 2003; Yang and Sharrocks, 2004).

A different mechanism by which SUMOylation can repress transcription is the re-

cruitment of the SUMOylated transcription factors in the repressive environment of subnuclear domains, such as the PML nuclear bodies. The PML (*promyelocytic leukemia*) gene was discovered in a chromosomal translocation that results in the fusion of PML protein with retinoic acid receptor and causes Acute Promyelocytic Leukemia (Salomoni and Pandolfi, 2002). Under normal conditions PML protein is found in nuclear bodies. PML is SUMOylated and it recruits many other proteins like Sp100, Daxx and p53 which can also get SUMOylated. SUMOylated Sp100 can interact with Heterochromatin protein 1 (HP1) in vitro and therefore it is possible that the recruitment of chromatin modifying enzymes in these nuclear bodies changes chromatin to a repressed state locally (Seeler et al., 2001).

DNA repair

SUMOylation has been found to play a role in DNA damage repair as well. The thymidine DNA glycosylase (TDG) is an enzyme that has an important function in the repair of mismatched bases in DNA. TDG removes the thymidine or uracil base from the mismatch with Guanidine and subsequently a downstream enzyme repairs the abasic site (Hardeland et al., 2001). Non-SUMOylated TDG in vitro completes one round of reaction and then it binds to the product. SUMOylation of TDG results in reduced affinity of the enzyme for DNA structure and therefore TDG is able to complete multiple rounds of reaction in vitro. Thus, it seems that SUMO modification of the TDG enzyme is required for the dissociation of TDG from DNA after the base removal so that the next enzyme will be allowed to repair the gap (Hardeland et al., 2002).

SUMOylation appears to regulate also Topoisomerase I which is a DNA repair enzyme that is implicated in the recognition of DNA lesions (Liu et al., 2000). Another enzyme that is controlled by SUMOylation is the PCNA (proliferating cell nuclear an-

tigen). SUMOylation of PCNA during S phase inhibits the recombination repair of DNA. SUMO modification of PCNA probably ensures that DNA repair will not take place in the wrong phase of the cell cycle (Pfander et al., 2005) .

Subcellular localization

SUMOylation has been shown to induce changes in the subcellular localization of target proteins. RanGAP1 that was the first protein on which SUMO was found to be attached is involved in nuclear transport of proteins. RanGAP changes localization upon SUMOylation, as unmodified protein is cytoplasmic while SUMOylated RanGAP1 protein translocates to nuclear pore complexes were it associates with RanBP2 (Mahajan et al., 1997; Matunis et al., 1996). During mitosis SUMO modified RanGAP1 gets recruited in mitotic spindle (Joseph et al., 2002).

As it is described above, PML SUMOylation is required for the localization of other proteins in PML nuclear bodies (Hay, 2005). Another example of SUMO-dependent translocation is the case of NEMO, the kinase regulator of IκB, which is localized to the nucleus upon fusion to SUMO (Huang et al., 2003). Also, the CtBP transcriptional co-repressor has been shown to lose its nuclear localization when its single SUMO acceptor site is mutated (Lin et al., 2003).

Signal transduction

SUMO conjugation influences signal transduction. It can affect the activity of transcription factors that are downstream of signaling pathways as mentioned above (Smad4, Elk-1, STAT-1, androgen receptor, GR). SUMOylation can also regulate the nuclear translocation of a transcription factor, as in the case of NFκB where SUMOylation prevents the degradation of its inhibitor IκBα which keeps NFκB in the cytoplasm (Desterro et al., 1998). SUMOylation can regulate signal transduction through

regulation of the activation of the receptor, as it has been shown for type I TGF-β receptor (Kang et al., 2008).

SUMOylation and development

It is observed that disruption of SUMOylation in yeast leads to cell cycle progression defects. Depletion of Ubc9 causes a cell cycle arrest and the majority of cells appear to be large budded with increased cell volume and a single nucleus. These cells have their DNA replicated and they arrested at G2/M (Seufert et al., 1995). Similarly, tetrad analysis of Uba2 depleted cells showed that these cells produce microcolonies of 50-100 enlarged cells (Dohmen et al., 1995). They report also that *Δuba2* microcolonies consisted of 30% of cells with no bud, 60% of cells with one large bud and 10% of cells with two buds (Dohmen et al., 1995). Another study describes also an identical phenotype for Aos1 depleted cells (Johnson et al., 1997). Upon tetrad analysis of *Δaos1* cells, the authors observe that the mutant cells grow for a few generations and form colonies of about 100 cells, but the cells eventually become enlarged and finally lysed (Johnson et al., 1997).

Despite the detailed phenotypic analysis in yeast, there is not enough information about the role of the SUMOylation pathway in the development of multicellular organisms. There are only a few studies that describe the developmental defects that are caused by the blockage of global SUMOylation.

In mice, an Ubc9 (SUMO E2) null allele causes early embryonic lethality (Nacerddine et al., 2005). The mutant embryos survive until the blastocyst stage and they undergo implantation in uterus, but they die afterwards. The authors suggest that the lethality could be attributed to the decline of maternal Ubc9 protein. Culture of the blastocysts *in vitro* showed that Inner Cell Mass (ICM) cells of Ubc9 mutant embryos

die through apoptosis, while the non-dividing trophoblastic cells remain alive (Nacerddine et al., 2005). When examined closely, the ICM cells of the mutant embryos had mitotic chromosome condensation and segregation defects.

In zebrafish, injection of a dominant negative form of Ubc9 protein into one-cell stage embryos leads to embryonic lethality and extensive cell death (Nowak and Hammerschmidt, 2006). Dominant negative Ubc9 protein produces a severe phenotype as it interferes with endogenous Ubc9 protein that is maternally supplied. In contrast, injection of antisense morpholino oligonucleotides against *ubc9* mRNAs at one-cell stage embryos causes defects later in development, such as eye and brain size reduction and craniofacial malformation (Nowak and Hammerschmidt, 2006). Ubc9-depleted cells exhibit lower proliferation rates and Fluorescence Activated Cell Sorting analysis (FACS) showed an increase in the percentage of cells with replicated DNA (4n), and a small fraction of cells with 8n DNA content (Nowak and Hammerschmidt, 2006). In this study they also demonstrate that cell differentiation is not affected in Ubc9 mutant fish.

SUMOylation in growth and patterning of Drosophila

In *Drosophila*, SUMOylation regulates signaling pathways that are related with pattern formation, through the activity of the transcription factors that are downstream in the signaling cascade. Transcription factor Medea (Smad4 homologue) is found to be a SUMOylation target *in vitro* and *in vivo* (Miles et al., 2008). Medea is a downstream effector of Dpp signaling pathway and, together with phosphorylated Mad, it translocates to the nucleus and it activates the transcription of target genes (Affolter et al., 2001). SUMOylation of Medea regulates negatively the Dpp signaling pathway as it promotes nuclear export of Medea and thus, it appears to regulate the duration and the

range of Dpp signaling (Miles et al., 2008). In addition, SUMOylation seems to enhance the transcriptional activity of Vestigial (*vg*) as shown by the genetic interaction between SUMO and Vg (Takanaka and Courey, 2005). Spalt (*sal*) and Spalt-related (*salr*) transcription factor are also found to be SUMO targets *in vitro* and it is shown that SUMOylation changes their subnuclear localization *in vivo* (Sanchez et al., 2010). Genetic interaction showed that SUMO enhances Spalt and Spalt-related activity in induction of vein formation in the wing (Sanchez et al., 2010). Further, SUMOylation has been found to regulate negatively Dorsal (NF-κB family) transcription factor, as in Ubc9 mutants nuclear Dorsal is high and the Toll pathway upregulated, resulting in overproduction of hemocytes (Chiu et al., 2005; Huang et al., 2005).

Mutations in *smt3* (SUMO) and *lwr* (E2) genes have shown that SUMOylation is essential for viability. Zygotic *smt3* mutant animals (*Drosophila* SUMO) are reported to die at the early second instar stage (Nie et al., 2009). Germline clones that remove maternal deposition of Smt3 resulted in failure to hatch for more than 70% of embryos and those that hatched died as first instar larvae (Nie et al., 2009). Also, RNAi knock down of SUMO (smt3) in the prothoracic gland showed that SUMOylation is required for metamorphosis. Knock-down animals exhibited prolonged larval life and inability to form pupa due to inefficient production of Ecdysone (Talamillo et al., 2008b).

Ubc9 (*lwr*) has various reported alleles that generate different phenotypes. One class of mutations (*semi*) that are caused by P-element insertions results in late embryonic or first instar larval lethality (Epps and Tanda, 1998). The phenotype is attributed to the inability of maternal transcription factor Bicoid to enter the nuclei during embryogenesis. Another class of alleles contains lwr^{4-3}, which was generated by P-element excision, and lwr^{5}, which comes from a point mutation. Both of these alleles are recessive lethal and they are able to suppress chromosome non-disjunction mutation during meiosis (Apionishev et al., 2001). These alleles also complement the *semi* alleles in

complementation tests. A different study reports a molecular null lwr^{13} allele that deletes the entire *lwr* locus (Xiuli, 2003). The homozygous lwr^{13} die at the early third instar larval stage, and based on genetic evidence the lwr^{4-3} and lwr^{5} alleles are classified as hypomorphs, as the trans-heterozygous animals with lwr^{13} die at the middle and late third instar larval stage respectively (Xiuli, 2003).

METHODS AND MATERIALS

Fly strains

The UAS-RNAi transgenic stocks for the *aos1* and *uba2* were obtained from Droso-

phila Genetic Resource Center (DGRC) in Kyoto. The UAS-RNAi lines for *ubc9* (*lwr*) were obtained from Vienna Drosophila RNAi Center (VDRC). The UAS-RNAi for *smt3* was kindly provided by Dr. Barrio (Talamillo et al., 2008b). Bloomington Stock Center was the source for the PBac-element insertion line in *aos1* ($aos1^{17744}$) and for the P-element insertion that was used to generate the *uba2* alleles.

Mosaic analysis

The mitotic clones were generated with the MARCM system. Female flies of the genotype: hs-flp, UASsrcEGFP; actGal4, UASGFP; FRT82BGal80 were crossed with male flies: 1) FRT82B, 2) FRT82B$aos1^{17744}$/ TM6B, 3) UAS*aos1*; FRT82B$aos1^{17744}$/ TM6C, 4) UASp35; FRT82B and 5) UASp35; FRT82B $aos1^{17744}$/ TM6C for generation of GFP+ clones that are *wild type*, *aos1* mutant, cDNA-rescued *aos1* mutant, p35-expressing *wild type* and p35-expressing *aos1* mutant respectively. The progeny of each cross were heat shocked for 1 h at 37°C, about 48 hours after egg lay. The heat-shock induced expression of Flipase facilitates the mitotic recombination of the two FRT containing chromosome arms. The daughter cell that will obtain 2 copies of Gal80 will not express the UASGFP and UASsrcEGFP transgenes, as Gal4 will be inhibited by Gal80. The daughter cell that will obtain 2 copies of the non Gal80 FRT chromosome, and therefore it will have the desired genotype, it will be marked with the expression of GFP and will give rise to GFP+ clone cells after subsequent divisions. The larvae were dissected at late third instar larval stage, 2.5-3 days after heat shock. For the p35-expressing *aos1* mutant clones, the larvae were dissected at different time points (2.5-3.5 days after heat shock) in order to image the mutant cells in various steps of delaminating process. In these experiments the time of heat shock changed accordingly.

The MARCM system was used also for the generation of the *uba2* mutant clones. Female flies of the genotype: hs-flp, tubGal4, UASnlsGFP; FRT80BtubGal80 were crossed with male flies: 1) FRT80B, 2) FRT80B*uba2*^{N2}/ TM6C and 3) UAS*uba2*; FRT80B*uba2*^{N2} for generation of GFP+ clones that are *wild type*, *uba2* mutant and cDNA-rescued *uba2* mutant respectively. The clones are induced with heat shock of the progeny from each cross, 48 hours after egg lay. The larvae were dissected 2.5-3 days after heat shock, at late 3rd instar larval stage.

The mitotic clones in the *Minute* background were generated using the hsFLP-FRT system. Female flies of the genotype hs-flp; FRT82B or hs-flp; FRT82B*aos1*^{17744}/ TM6B were crossed with males FRT82B, ubiGFP, RpS3^{Plac92}/ TM6B for generation of GFP- clones that are *wild type* and *aos1* mutant respectively. The progeny of each cross were heat shocked for 1 h at 37°C, 4 days after egg lay (due to the developmental delay of *Minute* animals). The larvae were dissected 2.5 days after heat shock.

Plasmid construction

pBluescript SK+ -Aos1

The plasmid was generated in order to be used for in vitro translation of *aos1* and probe preparation for *in situ* hybridization. The cDNA clone LD33652 of *aos1* was digested with SacI-PstI and the *aos1* coding region fragment was cloned into SacI-PstI digested pBluescript vector, in orientation that T3 promoter of pBluescript vector would produce a sense RNA probe while T7 promoter would produce an antisense probe.

CMC105-*aos1* surrounding sequence

This plasmid was constructed in order to disrupt and replace *aos1* gene with homologous recombination. The first and most of the second exon of *aos1* was designed to be

deleted and therefore, the sequence that was cloned in order to be used for homologous recombination was upstream and downstream of the potential lesion. The upstream sequence of the targeted *aos1* region, which is extended up to the neighboring gene, was amplified with primers that contained restriction enzyme sites in their 5' end. The F primer contained a SpeI site and the R primer contained an AvrII site.

5' CCGACTAGTGAAACTGGTAATAGGTGCG F

5' CGCCTAGGACGCCTTTTGAAAATG R

After restriction enzyme digest, the amplified DNA sequence was cloned into a digested SpeI-AvrII CMC 105 vector.

In a second step, the CMC 105-upstream sequence plasmid was used as a vector for cloning the downstream sequence of the targeted *aos1* region, which contained the 3rd exon of *aos1*, part of the second exon and some of the neighboring gene sequence. The region was amplified with primers that contained NheI restriction site at their 5' ends.

5' CGAGCTAGCTGACACCATTTGCCG F

5' CGAGCTAGCGCGAAATAAGTCGGC R

After restriction enzyme digest, the amplified DNA sequence was cloned into a digested NheI CMC 105 vector-upstream sequence plasmid. Diagnostic digests allowed the distinction of clones that has the insert in the correct orientation.

pUAST AttB-Uba2 cDNA

This plasmid was used in order to make transgenic flies that express Uba2 under Gal4 driver control. The cDNA clone LD22577 of *uba2* was digested with BglII-XhoI and the *uba2* cDNA was cloned into BglII-XhoI digested pUAST AttB vector.

pCa4-AttB-Uba2 genomic region

The *uba2* genomic region construct was used to rescue the *uba2* mutant phenotype. The cloned genomic sequence included the *uba2* locus and the surrounding upstream

and downstream sequence up to the middle of the neighboring genes for each side. The genomic sequence was amplified with primers that contained restriction sites in the 5' end: F primer contained a PstI site and the R primer contained an XbaI site.

5' GCGCTGCAGTCACATACCTATCCG 3' F

5' CGTCTAGAAAGTGGAGCCAGGCGT 3' R

After restriction enzyme digest, the amplified DNA sequence was cloned into a digested PstI-XbaI pCa4-AttB vector.

In situ hybridization in larvae using RNA probe (Patel protocol)

1. Third instar larvae from w^{1118} fly stock were dissected in 1xPBS (137mM NaCl, 2.7 mM KCl, 10.14mM Na_2HPO_4, 1.03 mM KH_2PO_4). The anterior half of the larval body was inverted and the fat body was removed.

2. The dissected larvae were fixed with 4 % PFA in 1xPBS, for 20 min at Room Temperature.

3. Then, the larvae were rinsed in methanol 2-3 times.

4. Rehydration of the larvae followed, through rinses with different concentration of Methanol and PT (1xPBS, 0.2% Triton).

7 parts ME/ 3 parts PT 5 min at RT

5 parts ME/ 5 parts PT 5 min at RT

3 parts ME/ 7 parts PT 5 min at RT

FP (4% PFA in PT) 5 min at RT

5. Pretreatment.

a) 3 washes of the larvae for 5 min each in PTw (1xPBS, 0.1% Tween-20) on a rotator at Room Temperature

b) 1 wash of the larvae in Detergent/Tween-20 solution (1% SDS, 0.5% Tween-20,

50mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0 and 150mM NaCl) for 30 min on a rotator at RT

c) 1 wash of the larvae for 10 min in a 1:1 mix of SDS-Hyb (50% formamide, 5x SSC, 1% SDS and 0.1% Tween-20) and Detergent/Tween-20 on a rotator at RT

d) 1 wash of the larvae for 10 min in SDS-Hyb on a rotator at RT

e) Prehybridization in SDS-Hyb (+hs DNA) for about 1h at 65°C with agitation.

For 10 ml SDS-Hyb solution, 100 µl herring sperm DNA (from 10mg/ml stock) were added. The DNA was added after boiling it for 10 min to denaturate it.

6. Hybridization

RNA probe was diluted (1:25 to 1:200) in SDS-Hyb that had herring sperm DNA. The dilution varies depending on how strong is the signal that probe produces.

The diluted probe was boiled for 10 min and then cooled on ice.

The prehybridization buffer from larvae was removed and 100-200 µl of diluted probe per sample were added. The larvae were incubated in hybridization buffer overnight at 65°C.

7. Washes

a) 2 washes of the larvae for 20 min each in SDS-Hyb at 65 °C with agitation.

b) 2 washes of the larvae or 20 min each in 50 % SDS-Hyb /50 % Detergent Tween at 65 °C with agitation.

c) 2 washes of the larvae for 20 min each in 20 % SDS-Hyb /80 % PTw at 65 °C with agitation.

d) 2 washes of the larvae for 20 min each in Undiluted PTw at 65 °C with agitation.

e) 2 washes of the larvae for 20 min each in PT on a rotator at RT

8. The alkaline phosphatase conjugated anti digoxigenin antibody was diluted at 1:2000 in PT. The larvae were incubated with 200-400 µl of the diluted antibody solution on a rotator for 2 h at RT.

9. 4 washes of the larvae for 20 min each in PT on a rotator at RT

10. 3 washes of the larvae for 5 min each in AP buffer (5mM $MgCl_2$, 100mM NaCl, 100mM Tris-HCl pH 9.5 and 0.1% Tween-20) on a rotator at RT.

11. The larvae were incubated in dark with 0.5-1 ml BCIP/NBT solution (6.6 μl NBT and 3.3 μl BCIP/ ml AP) after the last wash with AP buffer. When the larvae were stained enough, the reaction was stopped by washing with PBS several times.

12. The tissues were mounted in 70 % glycerol in PBS.

Protein extraction from larvae and Western blot analysis

1. Every time, fresh lysis buffer was prepared: 1x RIPA (Pierce) in which 1x protease cocktail and PMSF in final concentration 1mM were added. PMSF is not stable in non organic solution for more than 30 min, so it should be placed last, just before the beginning of dissection. After preparation, the lysis buffer was placed on ice and it was divided to aliquots in different tubes (depending on the number of the samples).
In the case of a-SUMO Western blot, the lysis buffer that was used contained also NEM (*N*-Ethylmaleimide, SIGMA) protease inhibitor in final concentration 10mM, so that the SUMO deconjugation will be prevented. NEM was added also in the PBS solution that was used during dissection of larvae.

2. Larvae were dissected in 1xPBS (or 1xPBS + NEM). The anterior half of the body was inverted. The fat body and salivary glands were removed. In those samples that were *wild type* but needed to be compared with the mutant larvae samples, the imaginal discs were removed as well. The trachea, brain and cuticle were kept in all samples.

3. Each dissected larva was placed immediately in the tube with the lysis buffer. The lysis buffer was kept on ice all the time. The number of dissected larvae in the lysis

buffer depended on the needs of the experiment. Usually, high concentration protein samples have been prepared by placing 50 dissected larvae in 75 μl lysis buffer.

4. After finishing the dissection for each sample, a blue pestle was used to smash the larvae in the buffer. The grinding was continued till only the cuticle was remained.

5. The lysate was centrifuged in 15,700 x g at 4°C for 30min.

6. Then the supernatant was transfered in a new tube and it was placed on ice.

7. Loading buffer was added before the samples were applied to protein gel.

4x loading buffer (0.2M Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 10% BME, + Bromophenol Blue)

8. The samples were stored at -80°C or directly applied to the gel

9. Pre-cast gels from BIORAD were used (4-15 % acrylamide gradient gels for the a-SUMO and a-Aos1 Western blots and 7.5% acrilamide gels for the a-Uba2 Western blot).

10. For the blotting we used antiserum for SUMO, Aos1 and Uba2 that were kindly provided by Dr. Griffith (Long and Griffith, 2000) and were used at 1:500, 1: 5000 and 1: 1500 dilutions respectively. The level of loaded protein in each gel lane was monitored by re-blotting with an antibody for alpha-Tubulin (SIGMA) in 1:1000 dilution. The secondary antibodies, anti-Rabbit HRP (SIGMA) and anti-Mouse HRP (invitrogen) were used at 1:20000 and 1:5000 dilutions respectively. The blots were developed in autoradiography film, after incubation with chemiluminescent substrate (SuperSignal West Dura PIERCE and Immun-Star HRP substrate kit BIORAD).

Immunostaining

1. The 3rd instar larvae were dissected in 1xPBS and the anterior half of the larval body

was inverted.

2. The tissues were fixed in 4% PFA in 1xPBS, for 20 min at RT.

3. After fixation, the larvae were washed 3 times with 1xPBS

4. Incubation of the larvae in PBT (1xPBS, 0.2% Triton, 0.5% BSA) for 1h at RT with agitation.

5. The primary antibody was diluted in PBT and added in the larvae. Primary antibodies that were used for immunostaining : anti-cleaved Caspase 3 (1:500), anti-Tubulin (1:1000), anti-Cyclin A (1:500), anti-Cyclin B (1:500), anti-Elav (1: 80).

The larvae were incubated with the primary antibody overnight at 4 °C.

6. 3 washes of the larvae with PT (1xPBS, 0.2% Triton) for 10 min each at RT with agitation.

7. Incubation with the secondary antibody in dark for 2 h at RT

8. 3 washes of the larvae with PT (1xPBS, 0.2% Triton) for 10 min each at RT with agitation.

9. Imaginal discs were removed and mounted in 70% glycerol in 1xPBS

Imaginal disc cell preparation for FACS

1. 1.5 ml tubes were siliconized by adding 1.5 ml of sigmacote to the tubes for 5 minutes. Excess was removed and the tubes were left to air dry overnight.

2. Dissociation medium (90% 10x Trypsin-EDTA SIGMA, 1X PBS, + 0.5 μg/ml Hoechst) was prepared and placed in a siliconized tube.

The medium was placed on ice and kept on ice throughout the dissection process.

3. Larvae were transfered from vial/bottle with forceps into a Petri dish or glass crystallization dish. The larvae were rinsed with distilled water to remove food/yeast, then rinsed briefly with 70% EtOH, and after that rinsed twice with distilled water.

4. Three drops of 1x PBS were placed on a siliconized glass slide. In one drop the washed larvae were dissected. The anterior half of the larva was inverted and stripped of fat body and gut. Then the anterior half was transferred to the second drop, using forceps, to dissect away the wing imaginal discs, excluding other tissues e.g. other discs, trachea. Finally, the discs were transferred to the third drop to wash them of residual media (yeast) and larval tissues. In the third drop, using a needle the imaginal discs were cut in 3-4 pieces.

5. The cut discs (piles of 6-10 cut discs) were transferred into the dissociation medium via a 20ul micropipettor which has been coated with protein and fat from the dissected larvae by pipetting up and down the liquid from the first drop. Each sample contained about 40 discs.

6. When all the discs were collected and placed into the medium, the tube was removed from the ice and placed on rotator at room temperature. Dissociation was allowed to occur for ~1.5 h. Every 20 minutes, the medium was agitated by briefly vortexing (no more than 2 seconds).

7. Finally, the tube was removed from the rotator, placed on ice, and passed to the Cytometry facility for FACS analysis.

dsRNA knock-down in S2 cells

For the *aos1* dsRNA knock-down two different gene regions were targeted. Using the LD33652 cDNA clone of aos1 as template, the two different region of *aos1* were amplified with primers that contained a T7 promoter sequence in their 5' end:

5' TAATACGACTCACTATAGGGACAGTTGGCAGCACTGATTC 3' *aos1* F1

5' TAATACGACTCACTATAGGGCAGAGGTCTTCTCCTTCAAG 3' *aos1* R1

5' TAATACGACTCACTATAGGGGCGACCAACGAGGAACTGTT 3' *aos1* F2

5' TAATACGACTCACTATAGGGTAGAGCTTCGTCGCCCAGTA 3' *aos1* R2

The PCR products were purified and they were used as a template for in vitro translation with the Ambion Megascript T7 kit. The produced RNA was purified with RNeasy kit. Next, Tris-HCl pH 7.5 in final concentration 10mM and NaCl in final concentration 100mM were added in the purified RNA in order to achieve the correct salinity and pH, and therefore to avoid osmotic reaction when RNA is added to the S2 cells. The two strands of RNA were annealed by incubation in 95 °C for 5 min and then cool down at RT for 1h.

The same process was followed for the production of dsRNA that targets *smt3*. The gene region was amplified from the cDNA clone LD07775 with the primers:

5' TAATACGACTCACTATAGGGTTTGACCACTTAGCAGCTTCAACAA 3' *smt3* F

5' TAATACGACTCACTATAGGGACCATTTTCTTGTCTGCAAATG 3' *smt3* R

For negative control, we generated dsRNA from the pMT vector backbone sequence.

Prior to the knock-down experiment, the S2 cells were split to 1 million /ml and kept in 10% Fetal Bovine Serum (FBS) containing medium (Schneider's *Drosophila* medium). Two days later, the cells were split again to 2 millions /ml in S2 medium that had no serum. The cells were then placed in 6-well plates (1 ml per well) and they were left to attach to the bottom of the well for 20 min. After 20 min, 1 ml of 20% serum containing medium was added in each well. Immediately afterwards, the dsRNA was added in the cells (~10 μg in each well that contained 2 millions of cells). Every second day the dsRNA is renewed through the course of the knock-down treatment. The cells were collected at various time points and after cell lysis, the extract was examined through Western blot analysis in order to confirm the effect of knock-down in the protein levels.

Chapter 1

Analysis of the *aos1*^{17744} allele and generation of additional alleles

The *aos1*^{17744} mutation

In contrast with other genes of the SUMOylation pathway, the biological role of *aos1* in *Drosophila* has not been studied (Talamillo et al., 2008a), probably due to the unavailability of *aos1* mutations. At the beginning of this work, the only publically-available putative *aos1* mutation was a PiggyBac transposable element insertion in the 5'UTR of the *aos1* gene, 41bp before the ATG (Fig 6A). This transposable element mutation ($aos1^{17744}$) was one of several that were generated by the *Drosophila* gene disruption project (Thibault et al., 2004). The project's goal was the generation of gene mutations throughout the genome using transposable elements. The $aos1^{17744}$ insertion, although it was not a mutation in the coding region of *aos1* gene, caused elimination of the imaginal structures from developing larvae, an interesting phenotype that drew our attention to further analysis.

Phenotypic description of the $aos1^{17744}$ mutants

Flies that carry the $aos1^{17744}$ mutation as heterozygotes do not exhibit any obvious mutant phenotypes in the external adult cuticular structures or viability. However, the homozygous mutant animals die at the early pupal stage. Pupation in these animals generally occurs after a significant developmental delay compared with wild type animals (see graph in 1.8). The mutant pupae die before any adult structures are formed.

At the 3rd instar larval stage the homozygous mutants completely lack the entire set of imaginal discs. In Figure 6B the expression of Histone 2A-RFP fusion protein in *wild-type* animals illuminates the imaginal discs, which are characterized by high nuclear density and therefore easily distinguished from the larval cells. In contrast, we did not observe intense RFP staining in the anterior portion of $aos1^{17744}$ homozygous mutant larvae, indicating the absence or severe reduction of imaginal disc structures.

Another feature of the *aos1*^{17744} phenotype was the size difference of the CNS in the mutant animals. The CNS of the homozygous mutant larvae was smaller than comparable *wild-type* controls. Staining with Phalloidin that labels F-Actin at the cell cortex showed that the size difference was due to the loss of the optical lobes in the mutant CNS (Fig 6C, 6C').

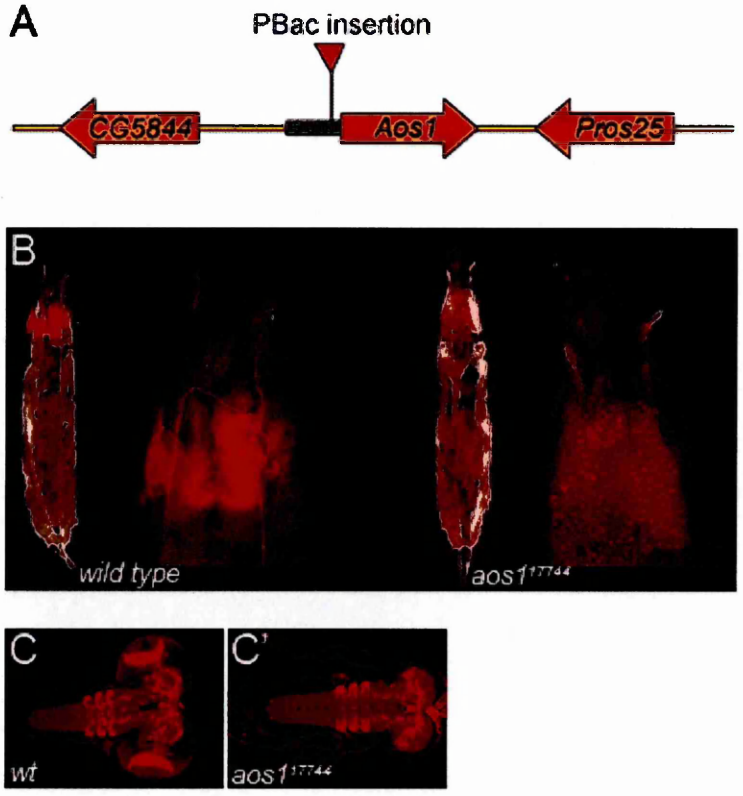

Figure 6
In *aos1*^{17744} mutant larvae, imaginal discs and optic lobes of CNS are absent.
A. The *aos1* gene model. The PBac transposable element that causes the *aos1*^{17744} phenotype is inserted in the 5'UTR of *aos1* gene.
B. Histone 2A-RFP expressing larvae. The imaginal discs in the *wt* 3rd instar larvae are visualized due to high nuclear density of these tissues. In contrast, the imaginal discs are absent in homozygous *aos1*^{17744} mutant larvae.
C and C'. The CNS of *wt* and mutant larvae stained with phalloidin. The mutant CNS is smaller than the wt due to the loss of optic lobes.

Lastly, we observed that melanotic tumors often appear within homozygous mutant larvae, although not in every animal. The melanotic tumor formation has been reported to take place in *lwr* mutant animals as well (Huang et al., 2005). Strikingly, apart from

the defects in the imaginal discs and CNS, there was no other obvious growth phenotype in the rest of the larval organs and tissues.

Rescue experiments confirm *aos1*^{17744} to be an *aos1* allele

In order to confirm that the *aos1*^{17744} transposon insertion mutation was an actual allele of *aos1*, various rescue experiments were performed. First, complementation tests with deficiencies that span the *aos1* genomic region showed that the lesion responsible for the imaginal disc elimination phenotype resides in the chromosomal location of *aos1*. Furthermore, precise excision of the PBac transposable element completely rescued the lethality and the growth defect in imaginal discs and CNS of the *aos1*^{17744} mutant animals (Fig 7A). This result indicates that the phenotype of the *aos1*^{17744} homozygous mutants was most likely caused by the PBac insertion in the *aos1* gene.

Finally, in order to verify that loss of *aos1* caused the lethality and growth phenotypes, a genomic rescue construct was designed. The genomic rescue construct included the *aos1* gene region and the flanking upstream and downstream genomic sequence, extended to the half part of each of the neighboring genes (Fig 7B). One copy of this genomic construct was sufficient to rescue the lethality and growth defects of the homozygous mutant animals (Fig 7C).

In summary, by showing that: **1**) The *aos*^{17744} insertion failed to complement chromosomal deficiencies in the *aos1* region; **2**) That excision of the PBac element reverted the *aos1*^{17744} phenotypes; and **3**) That a small genomic fragment containing only the complete *aos1* gene could rescue all phenotypes associated with the PBac insertion, these experiments confirm that the *aos1*^{17744} transposable element mutation is an *aos1* allele.

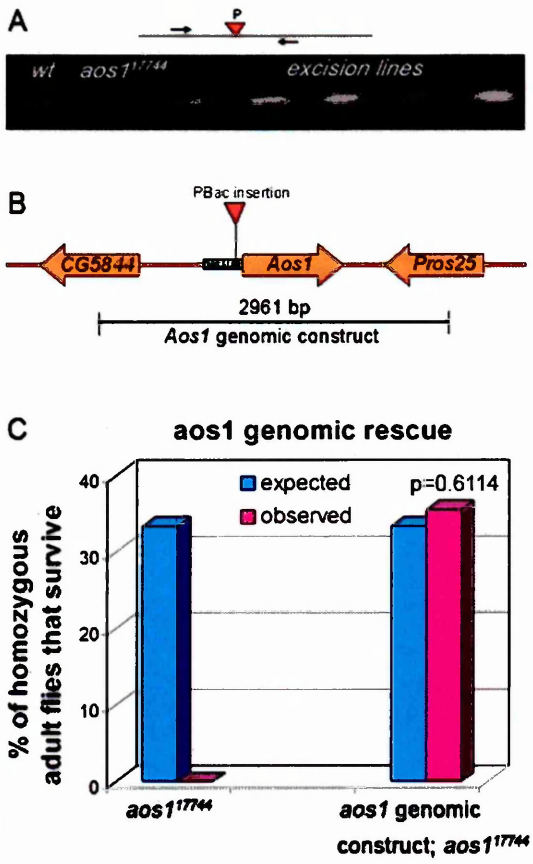

Figure 7
Rescues experiments confirmed *aos1*^{17744} mutation as an *aos1* allele.
A. The precise excision of the PBac transposable element is confirmed by PCR amplification. The primers bind on each side of the insertion site, so that the reaction produces a DNA band only when the transposable element is removed. The DNA that is amplified from the excision lines has the same size with the *wt* and thus the PBac is excised without altering the surrounding genomic sequence.
B. The Aos1 genomic construct includes the *aos1* gene region and its flanking sequence till the middle of the neighboring genes.
C. The graph shows the % of *aos1*^{17744} homozygous adult flies and the % of *aos1*^{17744} homozygous adult flies with at least one copy of the Aos1 genomic construct that survive. The percentage for the *aos1*^{17744} is 0% as the homozygous animals die as early pupae. The percentage of rescued mutant flies is very close to what is expected according to the Mendelian ratio for homozygous animals (1/3, as the homozygous balancer animals die at very early stage). Therefore, the rescue is complete.

aos1 is broadly expressed, but at variable levels in different tissues

The *aos1*^{17744} homozygous mutant animals primarily died at the pupal stage. At the 3rd instar larval stage the only affected tissues were the imaginal discs and CNS. This was surprising as the Aos1/Uba2 complex is known to be the only E1 SUMO activat-

ing enzyme in the organism and the E1 activity should be required for all SUMOylation events (Johnson, 2004). It is also evident from the literature that SUMOylation takes place in all tissues and at all developmental stages (Geiss-Friedlander and Melchior, 2007). Therefore, it was unexpected that *aos1* mutant animals did not exhibit more severe phenotypes, such as lethality in earlier developmental stages or defects in all or in the majority of larval tissues due to problems with cell viability or homeostasis.

As a starting point to understand the phenotype, *aos1* expression levels were examined in different tissues. *In situ* hybridization showed that *aos1* mRNA was expressed in the nurse cells of the ovaries and was maternally deposited in the embryo (Fig 8A). This observation is consistent with the results of other studies, which show that there is maternal deposition for all the SUMO pathway enzymes (Hashiyama et al., 2009). Later in embryogenesis the expression of *aos1* becomes restricted to the CNS and the gonads (BDGP expression data). In 3rd instar larvae, high levels of *aos1* expression were observed in the imaginal discs and the CNS (Fig 8B, 8C). The rest of the anterior-half larval tissues that were used for *in situ* hybridization (trachea, larval epidermis, salivary glands) did not exhibit strong *aos1* expression. However, Western blot analysis showed that there was *aos1* expression in these tissues as well (see below in Fig 11) but probably in lower amounts that were not detected by *in situ* hybridization.

The maternal deposition of *aos1* mRNA in $aos1^{17744}$ mutant embryos could explain the survival of the mutant animals through the early developmental stages. When maternal *aos1* was eliminated by generation of $aos1^{17744}$ germline clones in adult females, the embryos failed to hatch (data not shown). In the 3rd instar larval stage, *aos1* seems to be expressed more in imaginal discs and the CNS, the same tissues that have growth defects in $aos1^{17744}$ mutant animals. However, the rest of the larval tissues where *aos1* is expressed at lower levels do not exhibit any phenotype. One possibility is that

aos1^{17744} was not a null allele, and thus the Aos1 protein still produced in the mutant animals was sufficient for viability of tissues that do not require high levels of Aos1.

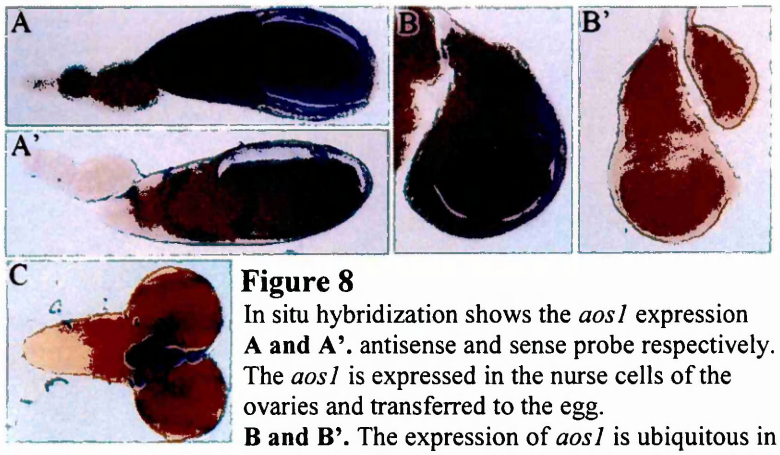

Figure 8
In situ hybridization shows the *aos1* expression
A and A'. antisense and sense probe respectively. The *aos1* is expressed in the nurse cells of the ovaries and transferred to the egg.
B and B'. The expression of *aos1* is ubiquitous in the imaginal discs. The sense probe in the B' is used as a signal specificity control.
C. The expression of *aos1* in the CNS is intense in certain zones. There is strong expression in the optic lobes, structures that are absent in *aos1* mutant animals.

aos1 transcription is detected in *aos1*^{17744} mutant larvae

The *aos1*^{17744} allele is caused by a PBac transposable element insertion in the 5'UTR of *aos1*, so it does not physically disrupt the coding region of the gene. In order to test whether *aos1* gene function is abolished by the PBac insertion, the *aos1* mRNA presence in the mutant larvae was examined by RT-PCR. A pair of primers was designed to amplify part of the *aos1* transcript sequence. As a control for genomic DNA presence in the sample, a second pair of primers was used to amplify sequence that extended from *aos1* to the neighboring gene (Fig 9A). The result showed that *aos1* mRNA was still detected in the mutant larvae, although in lesser amounts than were observed in the *wild-type* animals (Fig 9B).

The presence of *aos1* mRNA in mutant animals implies that some functional Aos1

protein could still be produced in *aos1*^{17744} mutant larvae. Indeed, the Pbac insertion is in the 5' UTR and there is no direct interruption of the *aos1* coding region. If some functional protein is still produced in the mutants, then *aos1*^{17744} is not a null allele, and a true null allele of *aos1* could be expected to cause a more severe phenotype in larva development. To address these issues would require the generation of additional alleles of *aos1* and observation of the resulting phenotypes.

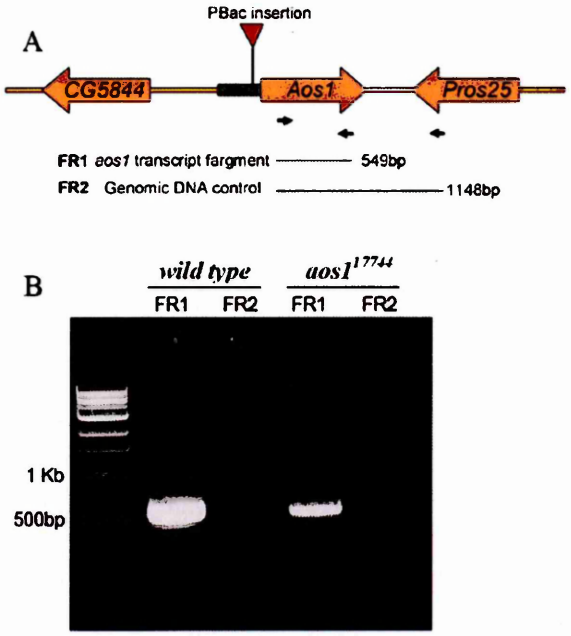

Figure 9
aos1 transcript can be detected in *aos1*^{17744} mutant animals with RT-PCR.
A. For the RT-PCR, two primers that bind in the coding region of *aos1* gene were designed. As a control for genomic DNA presence in the RNA samples, a second PCR reaction was set with a reverse primer that binds to the neighboring gene.
B. The *aos1* transcript is still present in the mutant sample, although it is less than the *wild type* (FR1 lanes). There is no genomic DNA contamination in either wt or mutant sample (FR2 lanes). This means that the PCR product in the FR1 lanes is amplified from transcript only and not from genomic DNA.

It is worth mentioning here that transheterozygotes carrying *aos1*^{17744} over deficiencies spanning the *aos1* genomic region did not exhibit more severe phenotypes than the *aos1*^{17744} homozygous mutant animals alone. This shows that further reduction of Aos1 protein has no additional effect in the phenotype. However, a complete elimination of Aos1 protein would be more conclusive about its function in fly development.

Identification of new *aos1* alleles by TILLing

In order to identify new alleles for *aos1*, the Seattle fly TILLing service was used. The fly TILLing service includes the screening of a large collection of mutagenized fly strains for mutations in a gene of interest; stocks of candidate mutations are then provided to the user for further analysis. The fly stock library consists of balanced lines that carry homozygous viable EMS mutations in the second and third chromosome, but in fact theses lines carry a lot more mutations (some of them recessive lethal) that have accumulated in the flies over time.

For the identification of mutations the genomic DNA of each line is isolated and mixed in pools of 8. The mixed DNA is used as a template for PCR amplification of the gene of interest with 5' end labeled primers. The PCR products are denatured and annealed so that the *wild-type* products will form double strands with the products that carry a gene mutation. The mismatches that are formed between the wild type and mutant strands are then recognized and cleaved by a single strand endonuclease. The denatured products of the endonuclease digestion are subsequently analyzed with gel electrophoresis. The pools that contain mutations for the gene exhibit more DNA bands in the gel that are of smaller size than the control. Then the DNA of the individual lines of these pools is analyzed separately in order to identify the mutant line and finally the mutation is sequenced and mapped (Winkler et al., 2005).

Many point mutations were acquired for the *aos1* gene through TILLing. Eleven of those point mutations resulted in amino acid substitutions and two of them in a premature stop codon (Table 1). Complementation tests with the $aos1^{17744}$ allele and with deficiencies that span the *aos1* gene were performed in order to identify which of these new mutations were *aos1* alleles. Unluckily, we found that all of the newly identified-mutations complemented the $aos1^{17744}$ and the deficiencies, except one.

The Q303* stop codon mutation failed to complement both the *aos1*^{17744} and two independent deficiencies. The animals that carried the Q303* mutation over *aos1*^{17744} or the deficiencies died as 3rd instar larvae or early pupae. Interestingly, those larvae had no imaginal discs and the CNS size was smaller than wild type, which is exactly the phenotype of the *aos1*^{17744} homozygous mutant animals. This could mean that the 30 amino acids that are missing from the C-terminal of the truncated Aos1 protein are important for the protein function or that the pre-mature stop codon results in nonsense mediated decay of the *aos1* mRNA (Nicholson and Muhlemann, 2010).

Unfortunately, although *aos1*^{Q303*} was found to be an *aos1* allele, the mutation was located in a degenerate TM6B balancer that lost the Tb marker, instead of the non-balancer chromosome which made it unsuitable to use for further studies. Also, another putative stop codon mutation, W29*, which was expected to cause a similar phenotype with *aos1*^{17744} and *aos1*^{Q303*} mutations, complemented the deficiencies and *aos1*^{17744}. Sequencing of the W29* line DNA showed that in fact there was not any mutation in the *aos1* gene and that the TILLing service sent the wrong fly stock. The correct fly stock was never found, as the genomic DNA isolate that was used for screening by Tilling had taken place a long time ago and the corresponding stock had likely been mislabeled or lost in the meantime.

amino acid substitution	Stop codon mutations
G46E	W29*
V74I	Q303*
Q82H	
G153D	
V181I	
P202L	
Q204K	
V207M	
R227Q	
G235S	
V296I	

Table 1.
aos1 mutations that were identified by Tilling

Generation of *aos1* null alleles by homologous recombination (Golic method)

As the TILLing service did not result in finding an *aos1* null allele that could be used

for further analysis, another approach was used to obtain it. In order to knock out the *aos1* gene by homologous recombination, the ends-out targeting method that was developed by Kent Golic and his group was applied (Gong and Golic, 2003).

The procedure of this method starts with the design and construction of a plasmid that includes the gene targeting homologous sequence flanking the *mini white* marker gene. The vector that was used (CMC 105) had already the *mini white* gene, one multiple cloning site at each end of the gene, one FRT sequence at the external end of each multiple cloning site and two P-element ends that flank all the above sequences. Approximately 1.7Kb of the genomic sequence upstream of *aos1* (starting from the 5'UTR of *aos1*) and about 1.8 Kb of the genomic sequence downstream of *aos1* (starting from the middle of the second exon of *aos1*) were cloned at the 5' and 3' ends of *mini white* gene respectively. The cloning design was such that the first and most of the second exon of *aos1* would be deleted and replaced by the *mini white* gene (Fig 10A).

In order to ensure also that no Aos1 protein would be produced after gene replacement, the ATG and some of the 5' UTR sequence of *aos1* was also omitted from the plasmid, and we confirmed that the remaining *aos1* gene coding sequence could not provide any open reading frame for Aos1.

Importantly, since the genomic sequence that was cloned in the plasmid contained the neighboring genes of *aos1*, the amplification of these regions was performed by a proof-reading polymerase so that no mutations in those genes would be induced by the homologous recombination. The cloned regions of the targeting plasmid were sequenced to confirm that there were no changes in the sequence during amplification.

The rest of the procedure for gene targeting was performed by Genetic Services, Inc. (Cambridge, MA). Briefly, the targeting plasmid was injected into fly embryos along with a helper plasmid encoding Transposase, and the P-element containing all of the

described sequences was thus inserted into the fly genome. Several transformant lines were obtained. These flies were subsequently crossed with flies that carry *hsp70-FLP* and *hsp70-I SceI*, and the progeny were heat shocked. The activated FLP in the progeny excises the target sequence-*mini white* cassette as a circular DNA fragment and the I SceI enzyme digests the circular DNA into a linear fragment. This is possible because there is a unique I SceI restriction site between the FRT sequence and the multiple cloning site upstream of the *mini white* gene. The linear DNA can then integrate into the genome after recombination with the homologous genomic sequence, thereby replacing the endogenous *aos1* gene.

The flies that emerge from this cross were selected based on the presence of *mini white*. The adults that have white or mosaic eyes are those in which the insertion has been excised and possibly reintegrated in the genome of some cells, while the colored eyed animals are those in which the insertion was not excised. Females with white or mosaic eyes, that hopefully carry some oocytes with reintegrated insertions, were crossed with males that express FLP constitutively to remove the initial insertion from the progeny so that only the reintegrated insertion will remain. The progeny of this cross were then selected for colored eyes and the individual males were crossed with *white* females for balancing and validation.

aos1 null alleles are obtained with homologous recombination (Golic method)

We obtained nine potential "*aos1* knockout" males from the Genetic Services, Inc. Fly lines were established after balancing the progeny of each of those males. Most of the lines were homozygous viable and complemented our original $aos1^{17744}$ allele. Some lines were homozygous lethal but they also complemented $aos1^{17744}$, indicating the insertion disrupted a different gene. Fortunately, two lines that were homozygous

lethal failed to complement the *aos1*^{17744} allele. Strikingly, the homozygous animals of these lines die at the 3rd instar larval stage, they lack imaginal discs and have a smaller CNS (Fig 10B).

In order to confirm the disruption of *aos1* in the new alleles, genomic DNA from homozygous mutant animals was isolated and the *aos1* genomic region was amplified by PCR and sequenced. Sequencing confirmed that both alleles have the correct gene sequence flanking the *mini white* insertion and therefore the *aos1* gene is deleted in the way it was designed. In addition, one copy of the *aos1* genomic construct was sufficient to rescue the lethality and growth defects of the homozygous mutant animals, verifying that the new mutations acquired with homologous recombination are real *aos1* alleles.

The new *aos1*^{Golic1,2} alleles exhibit the same growth phenotype as *aos1*^{17744}: the imaginal discs were missing in homozygotes and the CNS was smaller than the *wild-type*. Thus, although the *aos1* gene was deleted in the new alleles, the phenotype of the homozygous mutants was not more severe than the *aos1*^{17744} mutants. The only clear difference in phenotype was limited to the fact that *aos1*^{Golic1,2} mutants do not form pupae and they die as 3rd instar larvae, after a larval wandering period that lasted for about 10 days (Fig 10C). In contrast, the *aos1*^{17744} mutant animals formed pupae after a significant developmental delay (Fig 10C) and they mostly died as early pupae and rarely as 3rd instar larvae. The inability of *aos1* mutants to pupate normally is consistent with the reported requirement for SUMOylation in metamorphosis and in production of ecdysone (Talamillo et al., 2008b).

This slight difference in phenotype between the *aos1* alleles was not reflected in the Aos1 protein levels, since Western blot analysis showed Aos1 to be absent from all mutant alleles (Fig 11). The absence of Aos1 protein indicates that even *aos1*^{17744} is a very strong allele of *aos1* and equivalent in regard to imaginal disc growth to the

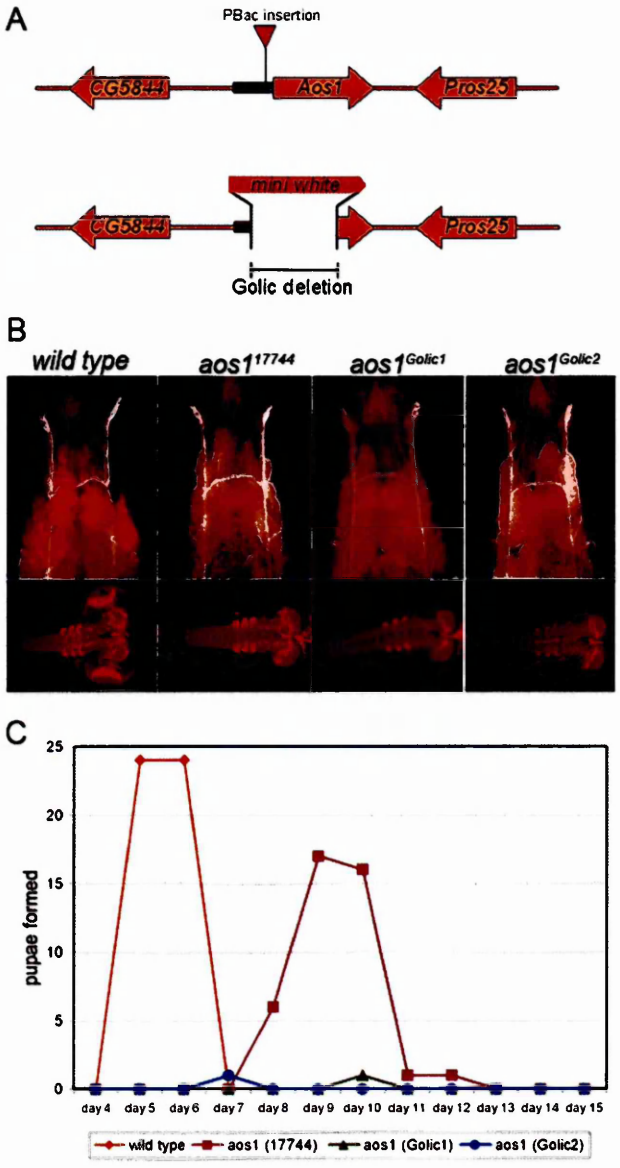

Figure 10

aos1^{Golic1,2} and *aos1*^{17744} mutant animals exhibited similar phenotypes

A. The deletion of *aos1* gene by homologous recombination (Golic method). The part of the gene that is deleted is the second half of the 5' UTR, the first exon and half of the second exon.

B. The phenotype of *aos1* mutant larvae for the different *aos1* alleles. The top panel shows Histone2-RFP expressing 3rd instar larvae. In the *wild type* larvae the imaginal discs are visible while in the aos1^{17744} and aos1^{Golic1,2} mutants the discs are absent. The bottom panel shows the phalloidin stained CNS of 3rd instar larvae for the different genotypes. The optic lobes are missing in all *aos1* mutants. It seems also that the ventral cord of the CNS of mutant animals is longer than the *wild type*.

C. The formation of pupa for the different *aos1* alleles. 4 days after the egg lay approximately 60 larvae for each genotype were transferred in a new vial and the pupa formation was monitored. We considered that pupa was formed when brown cuticle was generated by the animals (after the pre-pupal stage). The *aos1*^{Golic1,2} mutant larvae failed to form pupae and they die as 3rd instar larvae about 15 days after the egg lay. In two cases the *aos1*^{Golic1,2} mutant larvae generated cuticle only in the anterior body part, however these were counted as formed pupae (blue on day 7, green on day 10). The *aos1*^{17744} mutant larvae form pupae between day 8 and 10 after the egg lay. The *aos1*^{17744} mutants exhibit a significant developmental delay compared with the wild type animals that form pupae at day 5 and 6 after the egg lay.

aos1^{Golic1,} alleles. However, only *aos1*^{Golic1} and *aos1*^{Golic2} can be considered null alleles, since the *aos1* gene is deleted, no protein is detected, and a slightly more severe phenotype is observed.

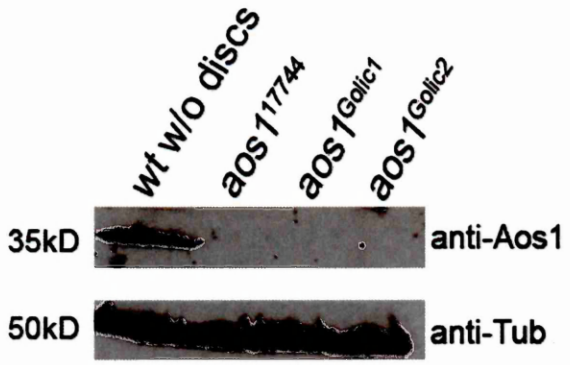

Figure 11
The Aos1 protein levels in the *aos1* mutant animals are monitored by western blot analysis. The larvae were dissected and the upper half of the body was used for protein extraction. The imaginal discs were removed from the *wild type* larvae so that the amount of protein in the wt sample will be comparable with the mutant ones, since the mutant larvae have no discs. The Aos1 protein is eliminated from all *aos1* mutant alleles. α-Tubulin is used for loading control.
Note here that in the *wild type* sample that has no imaginal discs there is significant amount of Aos1 protein, which comes from epidermis, trachea, muscles etc. In these tissues Aos1 protein is present although in situ hybridization could not detect *aos1* expression. This inability to detect *aos1* expression is probably due to limited sensitivity of the in situ hybridization technique.

Returning back to the initial question that led us to the generation of *aos1* null alleles, it is still not clear how the elimination of Aos1, which is necessary for all SUMOylation events in the organism, disrupts only a subset of cells in developing *Drosophila* larvae. A possible explanation may be that requirements for Aos1 are bypassed in the cells because Uba2, the other subunit of E1 SUMO activating complex, is still present. Although it is known that Aos1 and Uba2 participate in 1:1 ratio in the E1 complex (Long and Griffith, 2000), there is one study in yeast where it is observed that Uba2 is capable of interacting with itself (Dohmen et al., 1995). In this way, Uba2 could possibly provide the cells with activated SUMO that is sufficient for their survival. In order to test this possibility, it was essential to obtain *uba2* mutant animals and examine their phenotype in parallel with *aos1*.

Chapter 2

Generation of *uba2* alleles and double *aos1, uba2* mutant animals.

Generation of new uba2 alleles with imprecise P-element excision

For the *uba2* gene there are no available mutations. In the 5' end of *uba2* there are two EP transposable element insertions, but none of them affects the *uba2* gene and both are homozygous viable. However, these P-elements could be used to generate uba2 mutations by imprecise excision. The P-element insertion that was used for this purpose was P{EP}G4672, which is located in the 5' UTR of the *uba2* gene (Fig 12).

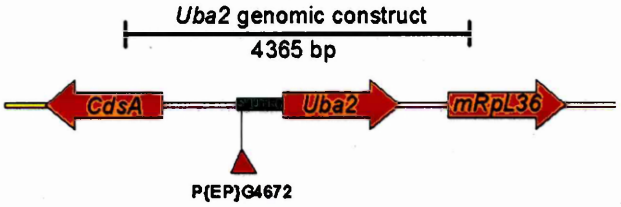

Figure 12
The *uba2* gene model. The transposable P-element that was used for imprecise excision is located in the 5'UTR of the *uba2* gene. For the genomic rescue of the *uba2* alleles that were obtained from the excision, a genomic construct was designed to include the uba2 gene region and the flanking sequence up to the 1/3 of the neighboring genes.

The fly lines that were acquired from the imprecise excision of the P-element were screened for lethality in homozygous animals. In this way our search was directed to the identification of strong *uba2* alleles. After isolating lethal excision lines, five putative *uba2* alleles were tested for the complementation of deficiencies that span the *uba2* gene region. Three of the lines failed to complement the deficiency.

In order to distinguish which of these three fly lines carried a mutation in *uba2* gene from those that carried mutations in other genes covered by the deficiency, we generated transgenic strains carrying an *uba2* genomic rescue construct. The genomic rescue construct included the *uba2* gene region and the flanking upstream and downstream genomic sequences, including half of each of the genes immediately neighboring *uba2* (Fig 12). The presence of one copy of this genomic construct was sufficient to rescue

the lethality of two of the three excision lines that failed to complement the deficiency. Thus, two new *uba2* alleles were obtained.

Interestingly, the homozygotes of both *uba2* mutants died as early pupae. At the 3rd larval instar stage, the imaginal discs were missing and the CNS was smaller than that of *wild-type* controls. The phenotype of the *uba2* mutants was therefore identical to that of the $aos1^{17744}$ mutant animals and the $aos1^{Golic1,2}$ mutants, with the exception of pupa formation.

Molecular characterization of the $uba2^{C3}$ and $uba2^{N2}$ alleles

The two new alleles, $uba2^{C3}$ and $uba2^{N2}$ were examined in order to verify the mutation in the *uba2* gene. The *uba2* genomic region of the mutants was amplified by PCR and sequenced. For both alleles the DNA fragment that was amplified by PCR had a bigger size than the control. This was an indication that the P-element was not excised completely and that some part of the transposon still remained in the genome. Sequencing showed that the *uba2* sequence was unaltered in any of the mutants, excepting the site of the P-element insertion in the 5'UTR of *uba2*. Thus, the excisions removed only internal parts of the P-element, leaving the *uba2* sequence intact. Nevertheless, this sequence rearrangement within the P-element was enough to cause *uba2* malfunction and produce a phenotype similar to *aos1* mutants. Therefore, since the coding region of *uba2* is not affected, there is probably a defect in the transcription or the translation of *uba2* in these mutants.

To test this hypothesis, the transcript levels of *uba2* in $uba2^{C3}$ and $uba2^{N2}$ mutant larvae were examined with RT-PCR. In both mutants *uba2* transcript was detected, although there was an obvious decrease in the transcript level compared with the control wild type animals (Fig 13A). In order to test whether the residual *uba2* transcript was

translated, the Uba2 protein levels were examined. Western blot analysis showed that Uba2 protein was eliminated in both mutants (Fig 13B). These results indicate that *uba2*^{C3} and *uba2*^{N2} are both very strong alleles of *uba2*, if not null. The fact that the *uba2* homozygous mutants can form pupae like the *aos1*^{17744} mutants, makes it likely that the *uba2*^{C3} and *uba2*^{N2} are not null alleles of *uba2* in the same way that that *aos1*^{17744} is not null allele of *aos1*. However, in terms of larval growth and development of imaginal structures, all the *aos1* and *uba2* mutants obtained are identical in the phenotype they produce.

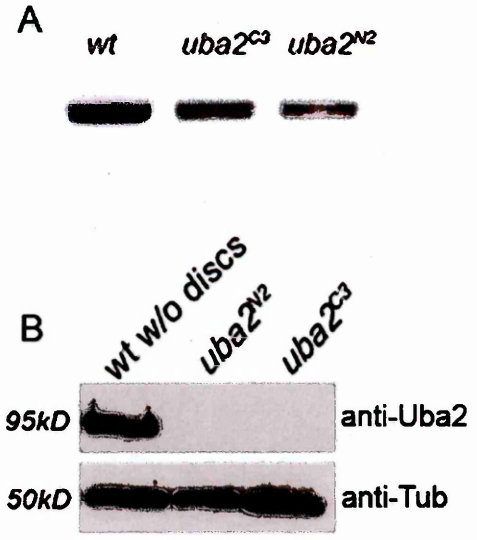

Figure 13
The *uba2* transcript and protein levels in *uba2* mutant animals were monitored with RT-PCR and Western blot analysis respectively.
A. RT-PCR showed that there is still *uba2* transcript in *uba2*^{C3} and *uba2*^{N2} mutants, although the transcript levels in both are decreased compared with the *wild type*. The DNA that is amplified in the RT-PCR is coming from transcript only and not from genomic DNA, because in that case the amplified fragment will have a bigger size as it will include the introns.
B. Western blot analysis was applied to monitor the Uba2 protein levels in the *uba2* mutant animals. The protein extract of dissected larvae was used for the blot. The imaginal discs from the wild type larvae were removed so that the wt sample will have comparable protein amount with the mutant ones which have no discs. The blot showed that Uba2 is eliminated form both *uba2*^{C3} and *uba2*^{N2} mutants and therefore the transcript detected by RT-PCR in the mutant animals probably is not translated.

Generation of double *aos1, uba2* mutant animals

uba2 mutant animals exhibited the same growth phenotype in imaginal structures as

aos1 mutants. In order to exclude the possibility that Aos1 and Uba2 can partially substitute for each other, a double *aos1, uba2* mutant was generated by recombination of the $aos1^{Golic1}$ allele with the $uba2^{N2}$ and $uba2^{C3}$ alleles. The double mutants that were obtained for all combinations of the above alleles had the same essential phenotype as the single mutants: the absence of imaginal discs in the homozygous larvae, a reduced CNS, and lethality at the 3rd instar larval stage. Hence, the phenotype of $aos1^{Golic1,2}$ null mutant animals was not enhanced by removal of Uba2. Consequently, the hypothesis that the *aos1* mutants have a defect in only a subset of larval tissues because Aos1 requirement could be bypassed with Uba2 presence is incorrect.

Global SUMOylation is severely reduced / eliminated in the mutant animals

The *aos1* and *uba2* mutant larvae have the same phenotype, which is not enhanced in the *aos1, uba2* double mutant animals. This finding is not only consistent with equal roles of Aos1 and Uba2 in the E1 SUMO activating enzyme, but it also unexpectedly indicates that the E1 complex is not required in every tissue. Therefore, either SUMOylation is not required in every cell of the organism or there is an alternative SUMOylation pathway that does not require Aos1 or Uba2 activity. In order to examine whether there is still SUMOylation in the mutant larvae, the global SUMOylation levels were monitored by Western blot analysis.

For these experiments, 3rd instar larvae were collected from all different mutant lines and w^{1118} as control. The anterior halves of dissected larvae were used for protein extraction in all cases. In the control sample, the imaginal discs were removed so that the amount of protein would be equivalent with the mutant ones in which the imaginal discs were absent due to loss of the E1 complex components. Western blots of these protein extracts with anti-SUMO antibodies showed that the global SUMOylation in

aos1 mutants, *uba2* mutants and *aos1, uba2* double mutants was nearly eliminated when compared with that in the *wild type* animals. Consistent with a failure to conjugate SUMO to substrates in the E1 complex mutants, we also observed that the free, unconjugated SUMO (approximately 15kD) was visibly increased (Fig 14). Also, these blots showed no major difference in SUMOylation levels between the different mutant genotypes, as all of them had the same reduction in bulk SUMOylation.

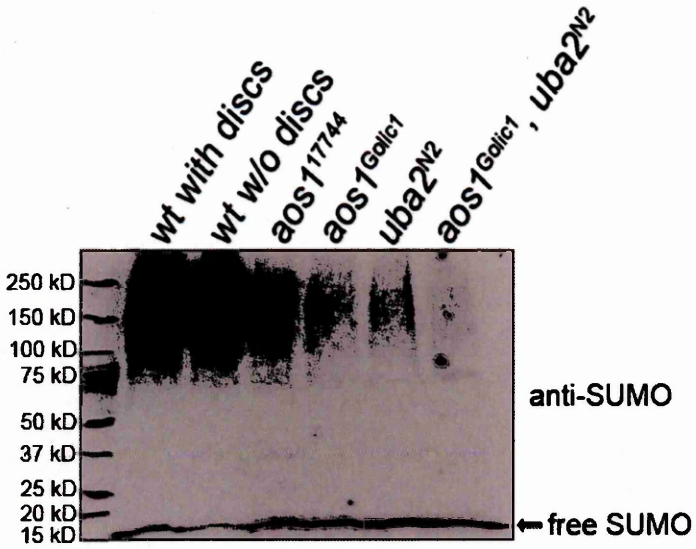

Figure 14
Western blot analysis showed that global SUMOylation in all mutants is severely reduced. At the same time the free, unconjugated SUMO is increased in the mutants compared with the *wild type* sample from which the imaginal discs are removed. The wt sample with the imaginal discs has more total protein and probably that is the reason that the free SUMO is more than the wt sample which has no imaginal discs.

These results confirm that if any of the E1 SUMO activating enzyme subunits is absent then the enzyme is not functional (Johnson et al., 1997). The requirement of both E1 subunits is also confirmed by the fact that the same phenotype is produced by the *aos1* and *uba2* mutations and the double *aos1, uba2* mutants. Secondly, these results verify that the E1 SUMO activating enzyme composed by the Aos1/Uba2 heterodimer is required for all SUMOylation events. The residual SUMOylation signal

that is observed in the mutants could be just non specific binding of the antibody. Alternatively, it could be SUMOylation that is facilitated by Aos1/Uba2 protein of maternal origin that has remained in the animals. Even in the cases of the *aos1*^{Golic1} and *aos1*^{Golic2} null alleles where there is no zygotic *aos1* protein, there is still the possibility of residual E1 activity provided by maternal protein. Last but not least, this result indicates that in those tissues where there is no obvious phenotype after Aos1/Uba2 removal, the global SUMOylation is severely reduced or eliminated. Strikingly, and unexpectedly, the elimination of bulk SUMOylation did not seem to affect either the homeostasis or survival of these cells.

In summary, it seems that SUMOylation is primarily required in only some cell types in developing larvae. Thus, the next experimental step was to understand the reason that these cells require SUMOylation more than other cells. For this purpose, the phenotype of imaginal disc cells upon the absence of Aos1 and Uba2 was examined.

Chapter 3

Phenotypic analysis of *aos1* and *uba2* mutant cells in the imaginal discs

Mosaic analysis of *aos1* and *uba2* mutant mitotic clones indicates that mutant imaginal disc cells die through apoptosis

In the homozygous *aos1* and *uba2* mutant 3rd instar larvae the imaginal discs are absent, preventing further analysis. In order to analyze the phenotype of the mutant imaginal disc cells in greater detail, the induction of mitotic clones with the hsFLP-FRT system was used (Golic, 1991). In particular, the MARCM technique was applied (Lee and Luo, 2001), so that the mutant clones could be distinguished by the expression of GFP.

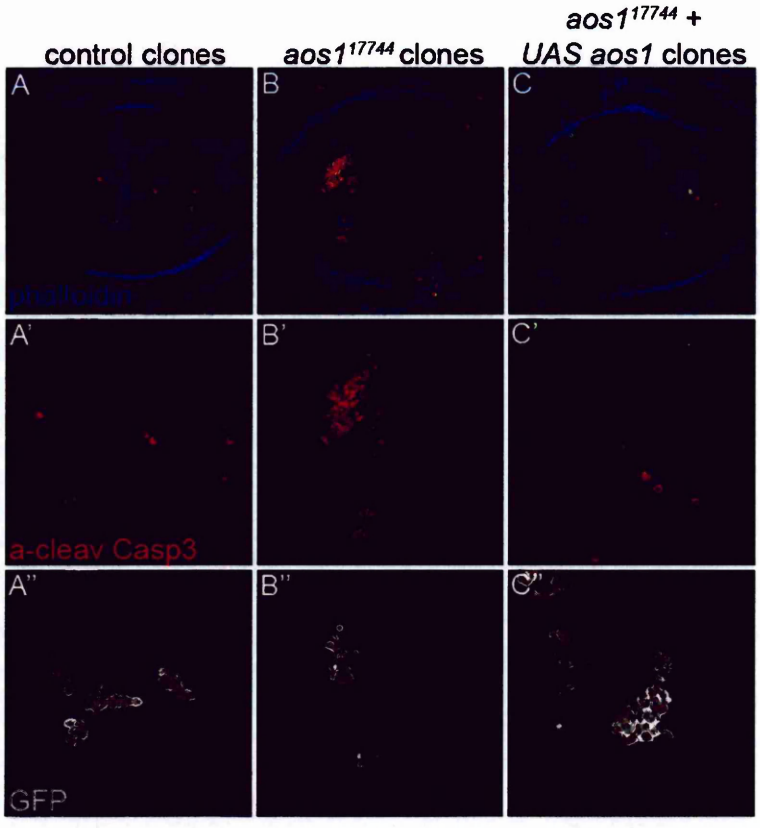

Figure 15
aos1 ^{17744} mutant mitotic clones die through apoptosis.
A, B, C. Mitotic clones positively marked with GFP and stained with a-cleaved Caspase3 (apoptotic marker) and phalloidin (F-actin staining).
A', B', C'. Magnification of the clones presented in A, B and C respectively. In the *aos1* ^{17744} mutant clones cells (B') the Caspase3 activation all over the clone indicates that these cells are massively dying through apoptosis. In contrast, apoptosis takes place randomly in *wild type* clone cells and non clone cells of the disc epithelium (A'). The expression of *aos1* cDNA in the mutant *aos1* ^{17744} cells rescues the lethality of these cells (C').
A", B", C". The GFP channel shows the outline of the mitotic clones in A', B' and C'. The *aos1* ^{17744} mutant clone shown in B" is disintegrated as the cells are dying.

Three days after induction, *aos1*^{17744} mutant clones in the wing imaginal disc epithelium appeared to be disintegrated. Staining with anti-Cleaved Caspase 3 antibody showed that in *aos1*^{17744} mutant cells, Caspase 3 was activated and thus the cells were dying through apoptosis (Fig 15B). This phenotype was a consequence of the absence of Aos1 protein, as the clone lethality was rescued by expression of the *aos1* cDNA in the mutant clones (Fig 15C).

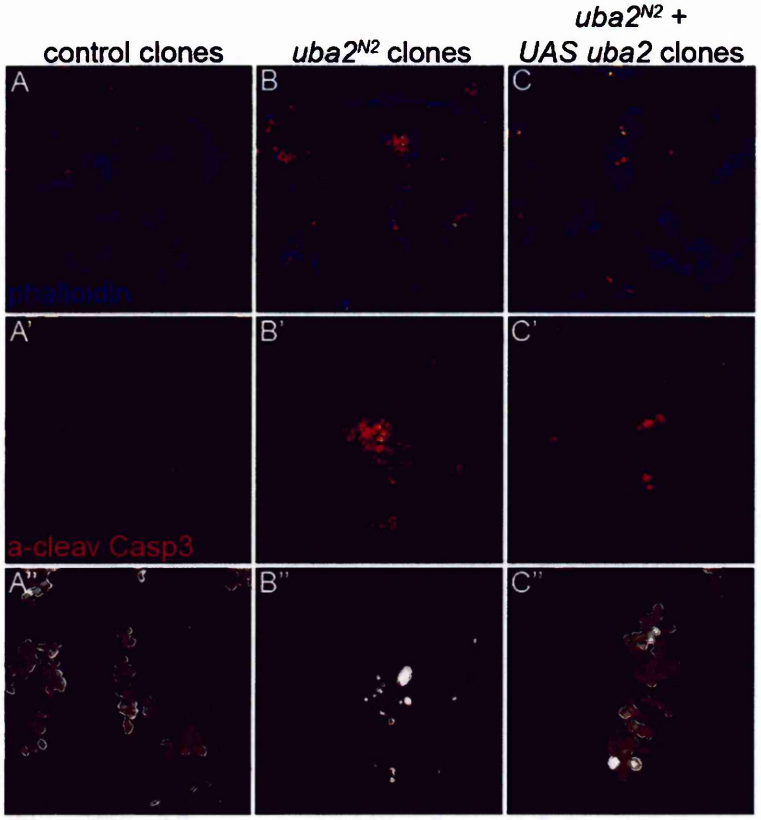

Figure 16
uba2^{N2} mutant mitotic clones die through apoptosis similarly with the *aos1*^{17744} mutant clones.
A, B, C. Mitotic clones positively marked with GFP and stained with a-cleaved Caspase3 and phalloidin.
A', B', C'. Magnification of the clones presented in A, B and C respectively. The presence of activated Caspase3 in the *uba2*^{N2} mutant clone cells indicates that these cells are dying through apoptosis (B'). The clone cell lethality is rescued with the expression of the *uba2* cDNA (C').
A", B", C". The GFP channel shows the outline of the mitotic clones in A', B' and C'. The integrity of the *uba2*^{N2} mutant clone shown in B" is disrupted as the cells are dying.

Similarly, *uba2*^{N2} mutant clones were induced in the wing imaginal disc epithelium and it was observed that *uba2*^{N2} mutant cells were also dying through apoptosis (Fig

16B). The lethality of these clones was fully rescued by the expression of the *uba2* cDNA (Fig 16C).

Expression of the p35 Caspase inhibitor in *aos1*^{17744} mutant disc cells reveals more mutant phenotypes

Since the *aos1*^{17744} mutant clone cells were dying through apoptosis, their phenotype could not be analyzed further. However, it is possible that there were more defects in those cells and apoptosis could be just a secondary consequence of these defects. In order to examine possible additional defects, the apoptosis in *aos1*^{17744} mutant cells was blocked by the expression of the p35 Caspase inhibitor (Best, 2008).

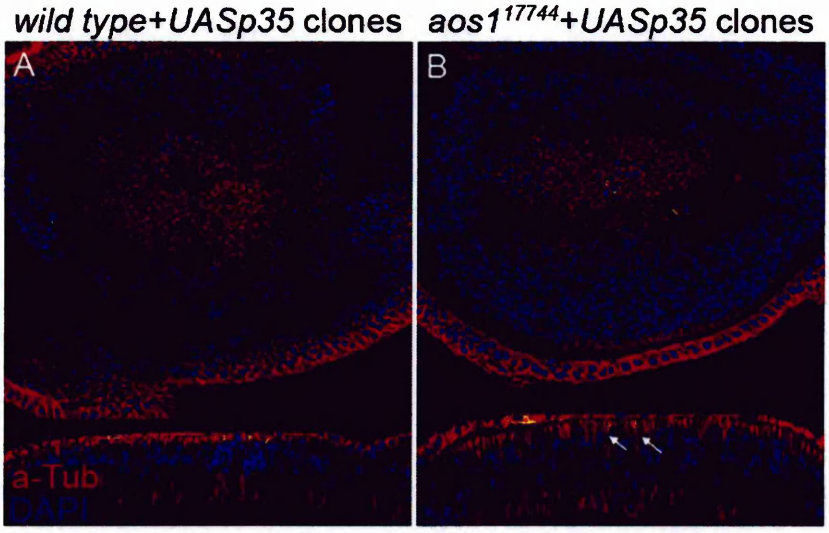

Figure 17
p35 expression in *aos1*^{17744} mutant clones reveals morphological defects in the mutant cells.
A. GFP marked wild type clones that express p35 Caspase inhibitor in the wing disc. In the cross section the clone cells that extend from apical to basal side are shown.
B. *aos1*^{17744} mutant clones that express p35 have aberrant morphology. In the cross section the *aos1*^{17744} mutant cells are shown to be detached from the apical surface and dropped in the basal side of the epithelium. Only thin membrane extensions from the mutant clones are still attached apically (white arrows).

p35-expressing *aos1*^{17744} mutant clones exhibited clear defects in their morphology. The cell bodies were detached from the apical surface of the epithelium and moved to the basal side, leaving only one or two thin membrane extensions reaching the apical surface. (Fig 17). Close observation of these clones showed that this was an intermediate stage of the extrusion process, as the mutant clone cells gradually detached from the apical side and dropped basally until they finally extruded from the epithelial monolayer as cell clumps on the basal surface of the wing disc (Fig 18).

This morphological change could be an outcome of the triggered cell death, as the phenotype is very similar to what is observed in dying epithelial cells of vertebrates (Rosenblatt et al., 2001). In this study they show that actin-myosin contractility in the dying cell and its neighbors push the dying cell to extrude from the epithelium before its death can cause a gap or leakiness in the epithelial sheet. The Actin accumulation on the apical side of the p35-expressing *aos1*^{17744} mutant clones (Fig 13A and A') suggests that this mechanism is also applied in *Drosophila*. In addition, Rosenblatt et al., (2001) show that the extrusion of dying cells precedes the Caspase activation and function, as Caspase inhibitors fail to prevent cell extrusion. This is true also for *aos1*^{17744} mutant cells, as p35 expression could not inhibit the extrusion of the clones.

As they age, p35-expressing *aos1*^{17744} mutant clones gradually disappear from the epithelium, which could mean that they still die eventually. Therefore, one possibility is that blockage of apoptosis by p35 expression is not complete and the cell death process is just delayed. An alternate explanation for the loss of p35-expressing *aos1* mutant clones is that they become completely extruded from the wing disc and then disperse within the larval body.

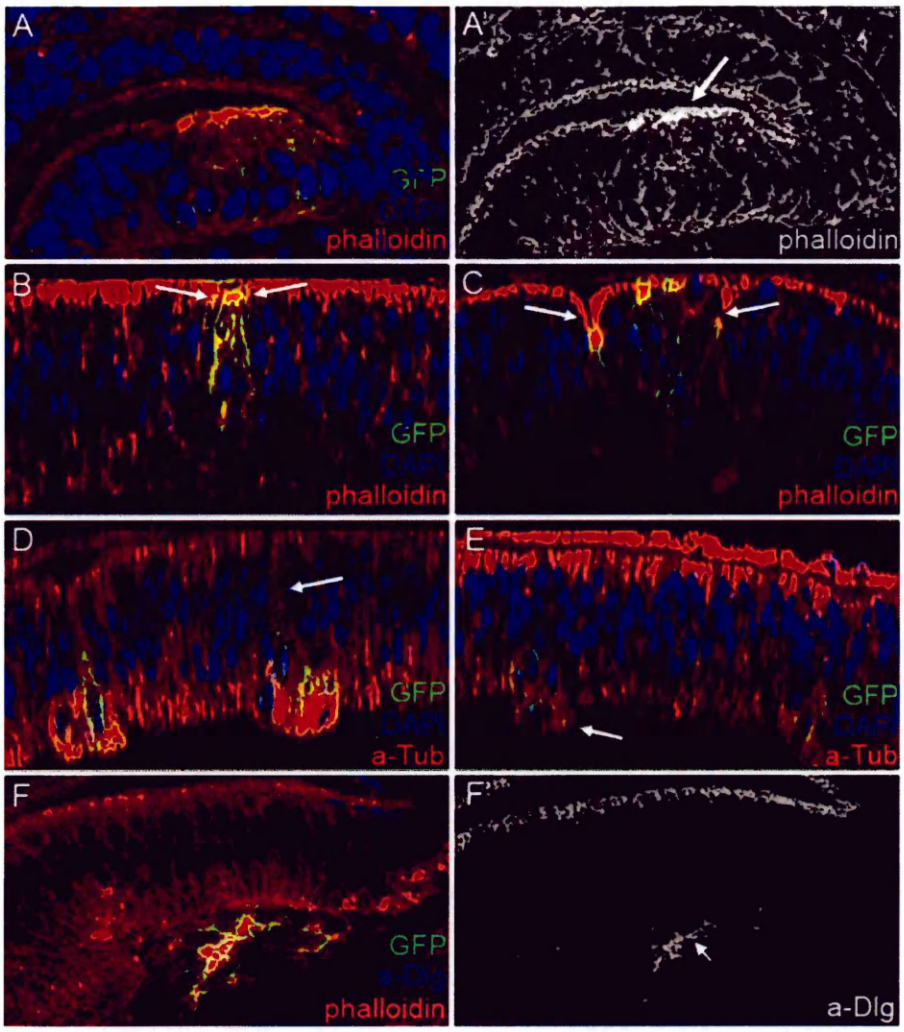

Figure 18

p35-expressing *aos1*^{17744} mutant clones gradually extrude from the epithelium

A and A'. Lateral side of a GFP marked mutant clone in the leg disc epithelium. The actin cytoskeleton is stained with phalloidin and the nuclei with DAPI. The red channel is shown in A'. There is an accumulation of actin in the apical side of the mutant cells (white arrow).

B, C, D, and E. Cross section of mutant clones in the wing disc epithelium. The apical surface of the mutant cells shrinks giving a triangle-like shape to the clone (B). Subsequently the clone cells get detached form the apical surface and they sink basally causing engulfment in the epithelium (left white arrow in C). The mutant cells are moving to basal side leaving only thin membrane extensions to the apical surface (D). Finally, the clone cells get completely detached from the apical side and start extruding from the epithelial monolayer (E).

F and F'. Lateral view of a mutant clone that has extruded from the wing disc epithelium. The clone cells retain their polarity as the Discs Large protein of septate junctions is still localized asymmetrically in the cell periphery with high accumulation in the surface that the mutant cells attach to each other (white arrow in F').

p35-expressing *aos1*^{17744} mutant clones exhibit cell cycle progression defects

Another interesting feature of the *aos1*^{17744} mutant cells in which apoptosis is blocked by the expression of p35 is that they exhibit impaired proliferation. As a result, the clones remain small in size but the cells themselves seem to be enlarged. In order to examine changes in the size and cell cycle profile of these mutant cells, the imaginal disc cells were dissociated and sorted by Fluorescent Activated Cell Sorting (FACS; Fig 19A).

FACS analysis showed that *aos1*^{17744} mutant cells accumulated at the G2/M phase (Fig 19B). The average cell size of these cells was also found to be larger than the *wild-type* control cells, but this was due to the high percentage of cells in G2/M, which skews the mean. Importantly, GFP^{+} *wild-type* cells that express p35 had no significant difference in their cell cycle profile compared with the GFP^{-} wild type cells, indicating that p35 expression alone did not interfere with cell cycle progression (Fig 19B).

Intriguingly, the accumulation of *aos1*^{17744} mutant cells in G2/M suggests that the DNA replication during S phase can be accomplished in the absence of Aos1 and consequently in the absence of SUMOylation. In order to provide more evidence that this is true, DNA synthesis was examined in the salivary glands of *aos1* homozygous mutant larvae. It is known that the cells in the salivary glands perform many endoreplication cycles, which means that they replicate their DNA without subsequent mitosis (Edgar and Orr-Weaver, 2001). We observed that the salivary gland cells of *aos1*^{17744} and *aos1*^{Golic1,2} mutant larvae were capable of incorporating EdU, confirming that DNA synthesis was not affected by loss of Aos1 (Fig 19C).

Our findings on cell cycle progression defects in *aos1* mutant cells are consistent with reports on *ubc9* (SUMO E2) mutant cells in yeast and zebrafish (Nowak and Hammerschmidt, 2006; Seufert et al., 1995). Removal of the E2 SUMO conjugation

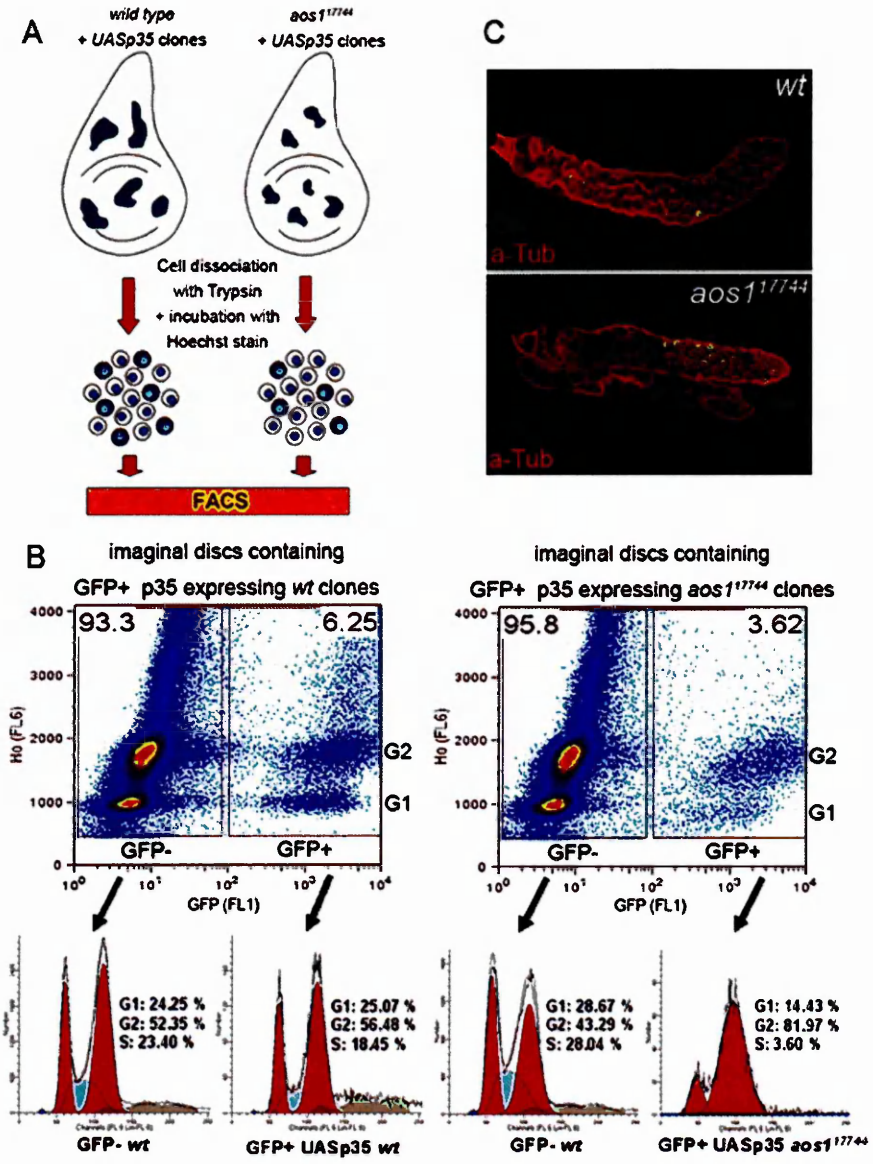

Figure 19

p35-expressing *aos1*^{17744} mutant clone cells accumulate at the G2/M

A. Imaginal discs containing GFP marked p35-expressing wild type control clones and GFP marked p35-expressing *aos1*^{17744} clones were treated with Trypsin to dissociate the cells. The dissociated cells were also stained with Hoechst DNA dye and then they were sorted by FACS.

B. FACS was used to sort the disc cells based on GFP expression and DNA content. The clone cells that were marked with GFP expression represented only a small percentage of the disc cells (6.25% and 3.62% for wt and mutant clones respectively in this example). Hoechst stain allowed cell sorting according to DNA content for each cell population and the distribution was modeled to calculate the percentage of cells in the different cell cycle phases (bottom panels). The *aos1*^{17744} mutant cells exhibit an accumulation in G2/M (last panel), which is also obvious in the heat map above. The cell cycle profile of the p35-expressing *wild type* clone cells is similar to the profile of the GFP- wild type non clone cells, indicating that p35 expression does not interfere with cell cycle progression.

C. Salivary glands of wild type and *aos1*^{17744} mutant larvae were incubated with EdU and stained with anti-Tubulin. The mutant salivary gland cells are capable of incorporating EdU in their DNA suggesting that endoreplication cycles are not affected in the absence of Aos1.

enzyme Ubc9 from yeast cells result in the accumulation of mutant cells in G2/M. Similarly, the *ubc9* mutant cells in zebrafish accumulate in G2/M and a small percentage of them undergo a second round of DNA replication. Collectively these observations indicate that SUMOylation *in vivo* is required for cell cycle progression and particularly for the passage through mitosis.

Induction of *aos1*^{17744} mutant clones in *Minute* background could not rescue the lethality of mutant cells

The FACS analysis above showed that *aos1*^{17744} mutant cells have defects in cell cycle progression, raising the possibility that the apoptosis of these cells was due to cell competition, a phenomenon where slow-dividing cells are killed by their wild-type neighbors (Morata and Martin, 2007). In order to eliminate cell competition, *aos1*^{17744} mutant cells were induced in a *Minute* /+ genetic background. *Minute* are a class of dominant mutations in the ribosomal protein encoding genes which cause a developmental delay and slow cell division (Dunn, 1937; Lambertsson, 1998). The growth advantage of *wild-type* cells against the mutant clone cells is removed when the clone cells are surrounded with *Minute* -/+ cells instead of *wild-type* cells, leading to the alleviation of cell competition and subsequent apoptosis of the "weak" cells.

In the case of *aos1*^{17744} mutant clones, the *Minute* /+ background did not prevent apoptosis of the mutant cells. We observed that Caspase 3 was still activated in the GFP-negative mutant clones. In discs containing control clones in the same Minute background, we only observed activated Caspase3 in the Minute /+ cells, as the *wild-type* cells outcompeted them (Fig 20).

The reason that the *aos1*^{17744} mutant cell lethality was not rescued in the *Minute* genetic background could be that: **1**) Cell competition was not fully eliminated; or **2**)

aos1 mutant cells are simply incapable of dividing and die as a cell-autonomous consequence of this defect. In the first case, *Minute*/+ cells can still divide, though slowly, while in *aos1*^{17744} cell cycle appears to be blocked at G2/M and therefore *Minute* cells still have a growth advantage between the two cell populations. Alternatively, apoptosis may be triggered internally in the *aos1*^{17744} mutant cells as a result of the cell cycle arrest. These two possibilities of internal or external trigger of apoptosis are not mutually exclusive, as both could take place at the same time in the *aos1*^{17744} mutant cells.

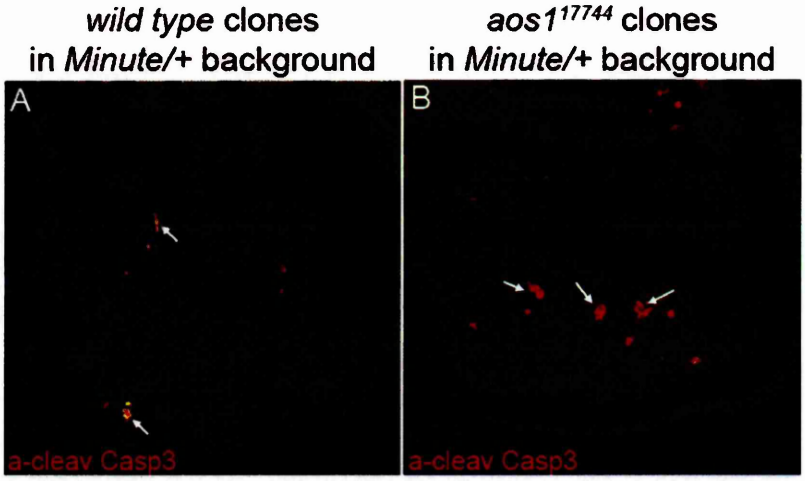

Figure 20
wild type and *aos1*^{17744} mutant clones in *Minute*/+ genetic background
The mitotic clones are marked with absence of GFP and the discs are stained with anti-cleaved Caspase3 to monitor apoptosis.
A. In the wild type control clones the apoptosis is restricted to the *Minute*/+ neighboring cells (white arrows), as the wild type cells clone cells outcompete them.
B. In contrast, the *aos1*^{17744} mutant clone cells are dying through apoptosis and therefore the lethality of these cells is not rescued by the induction of clones in *Minute*/+ genetic background.

Cyclin A and Cyclin B are stabilized in p35-expressing *aos1*^{17744} mutant clones

The accumulation of p35-expressing *aos1*^{17744} mutant cells in G2/M revealed by FACS analysis raised the question of what causes this defect in cell cycle progression. One possibility is that DNA damage activates the G2 checkpoint, as SUMOylation is

known to participate in DNA repair (Johnson, 2004) and its absence could affect the process of repair. The presence of double strand break foci in the mutant clones was examined by immunostaining with an antibody that recognizes the γ-phosphorylated H2Av Histone variant (Madigan et al., 2002). The result was negative, as the number of double-stranded break foci that were detected in mutant cells was similar to that detected in control *wild-type* clone cells (data not shown).

Subsequently, we examined the presence of mitotic Cyclins in the mutant cells, as the accumulation of these Cyclins is necessary for the G2/M transition and entry in mitosis (Budirahardja and Gonczy, 2009). The p35-expressing $aos1^{17744}$ mutant clones in the eye disc epithelium were used for this analysis. In the eye disc there is a distinct population of cells arrested in G1 (morphogenetic furrow). The morphogenetic furrow moves from the posterior to anterior side of the disc as a wave. In front of the morphogenetic furrow the cells divide in an asynchronous manner while behind the furrow the cells undergo one synchronous round of division and then they differentiate (Lee and Orr-Weaver, 2003). Therefore, the mitotic Cyclins are expressed on both sides of the morphogenetic furrow but not in the cells of the furrow itself (Fig 21A). Thus, we used immunohistochemistry to test whether Cyclin A and Cyclin B were present in mutant clones cells within the morphogenetic furrow.

Control, *wild-type* clones found in the furrow did not express Cyclin A since they were arrested in G1 like their neighboring cells outside of the clone. In contrast, all $aos1^{17744}$ mutant clones found in the furrow had high levels of Cyclin A in comparison with their neighboring wild type cells (Fig 21B). Similar results were obtained for Cyclin B expression, as half of the mutant clones in the morphogenetic furrow exhibited high Cyclin B levels (Fig 21C; n=6/12 clones in the furrow).

The presence of Cyclins A and B in the $aos1^{17744}$ mutant clone cells confirms the FACS result, which showed that mutant cells accumulated at G2/M. Also, it indicates

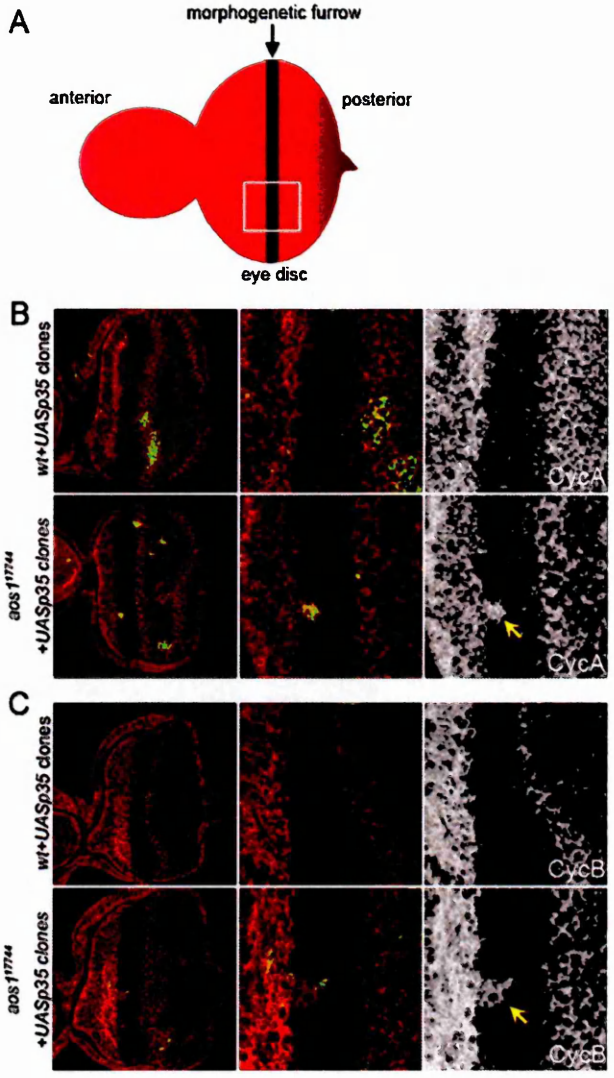

Figure 21

Cyclins A and B are stabilized in p35-expressing *aos1*^{17744} mutant clone cells

A. Schematic representation of the CyclinA and B expression in the eye imaginal disc. The morphogenetic furrow moves from the posterior to anterior of the disc. In the furrow the cells are arrested in G1 and they do not express Cyclins. In the anterior side of the furrow the cells are dividing asynchronously, while behind the furrow the cells complete one round of synchronous division and they differentiate. Therefore, expression of Cyclins A and B can be detected in cells in both sides of the furrow.

B. Left panels: GFP-marked, p35-expressing wild type and *aos1*^{17744} mutant mitotic clones in the eye imaginal disc epithelium. The discs are stained with anti-CyclinA (red). Middle and right panels: Magnification of mitotic clones that are found in the mophogenetic furrow. The wild type clones crossing the furrow have no CyclinA expression. In contrast *aos1*^{17744} mutant clones cells in the furrow have high CyclinA levels, as it is shown in the red channel on the right (yellow arrow). High CyclinA levels were observed in all *aos1*^{17744} mutant clones in the furrow that were examined.

C. GFP-marked, p35-expressing wild type and *aos1*^{17744} mutant mitotic clones in eye discs stained with anti-CyclinB (red). Similar to the observation for CyclinA, the *aos1*^{17744} mutant clone cells in the furrow showed high levels of Cyclin B (yellow arrow in the bottom right panel). Elevated CyclinB levels were detected in half of the *aos1*^{17744} mutant clones that were found in the furrow.

that the limiting step for progression through mitosis in the mutant cells is not a lack of mitotic Cyclin accumulation. In contrast, the high levels of Cyclins A and B in *aos1* mutant cells suggest the stabilization of these proteins. In yeast, Ubc9 (SUMO E2) depletion also causes the stabilization of type B Cyclins (Seufert et al., 1995), therefore our results suggest that the mechanism by which SUMOylation regulates cell cycle progression could be highly conserved.

The stabilization of Cyclins A or B can cause a delay/arrest in metaphase or anaphase respectively (Jacobs et al., 2001). However, no cells in metaphase or anaphase were observed in the $aos1^{17744}$ mutant clones. This could mean that there are additional defects in those mutant cells which can cause cell cycle arrest before metaphase, and Cyclin A and B normal degradation would not rescue cell cycle progression. The identification of these possible additional defects in the $aos1^{17744}$ mutant cells remains an open question.

SUMOylation pathway components are required in dividing but not differentiated imaginal disc cells

The results obtained from the phenotypic analysis of $aos1^{17744}$ mutant cells in the imaginal disc epithelium indicate that Aos1 elimination caused a cell cycle arrest in G2/M. Thus, DNA replication (S phase) seems to be unaffected in the mutant clone disc cells. Consistent with this, DNA synthesis was normal in the salivary glands of $aos1^{17744}$ homozygous mutant larvae. Thus, it is possible that Aos1 and subsequently SUMOylation are not required in the endoreplicating cells, but only in the dividing cells. This could explain the fact that *aos1* and *uba2* mutant larvae exhibit defects only in imaginal discs and the CNS which are composed of dividing cells, while the cells in the rest of larval tissues endoreplicate and they grow in size but they do not divide. It

has been shown that several mutations that inhibit cell cycle progression cause late 3rd instar larval-early pupal lethality and defects in imaginal discs (Gatti and Baker, 1989). The fact that *aos1* and *uba2* mutants exhibit a very similar phenotype implies that these genes function primarily in cell cycle progression.

If this is the explanation of the observed phenotype in *aos1* and *uba2* mutants, it would imply that SUMOylation is not required for the survival or the homeostasis of non-dividing cells. In order to test this possibility, the effects of Aos1 and Uba2 depletion was examined in dividing and differentiated cells of the same tissue- the eye imaginal disc. The *GMR-Gal4* driver was used for the expression of RNAi only in differentiated cells of the eye disc (behind the morphogenetic furrow), and the *Eyeless-Gal4* driver was used for RNAi expression in both dividing and differentiated cells of the eye disc.

Surprisingly, the knock down of *aos1* and *uba2* in differentiated cells had no effect on the eye imaginal disc or adult eye formation, while the expression of the same RNAi lines with the *Eyeless-Gal4* driver resulted in a dramatic reduction of the imaginal disc size and subsequently in a small adult eye (Fig 22). In many cases the phenotype was so severe that there was no head formation and the adult flies never eclosed. We observed similar phenotypes following RNAi knockdown of *ubc9* (SUMO E2) and even the *sumo* gene itself (Fig 22), indicating that disruption of proliferative growth but not cell viability is a common effect of disrupting the SUMOylation pathway. Taken together, these results indicate that *in vivo* SUMOylation is primarily required for cell division and is dispensable for cell survival or homeostasis.

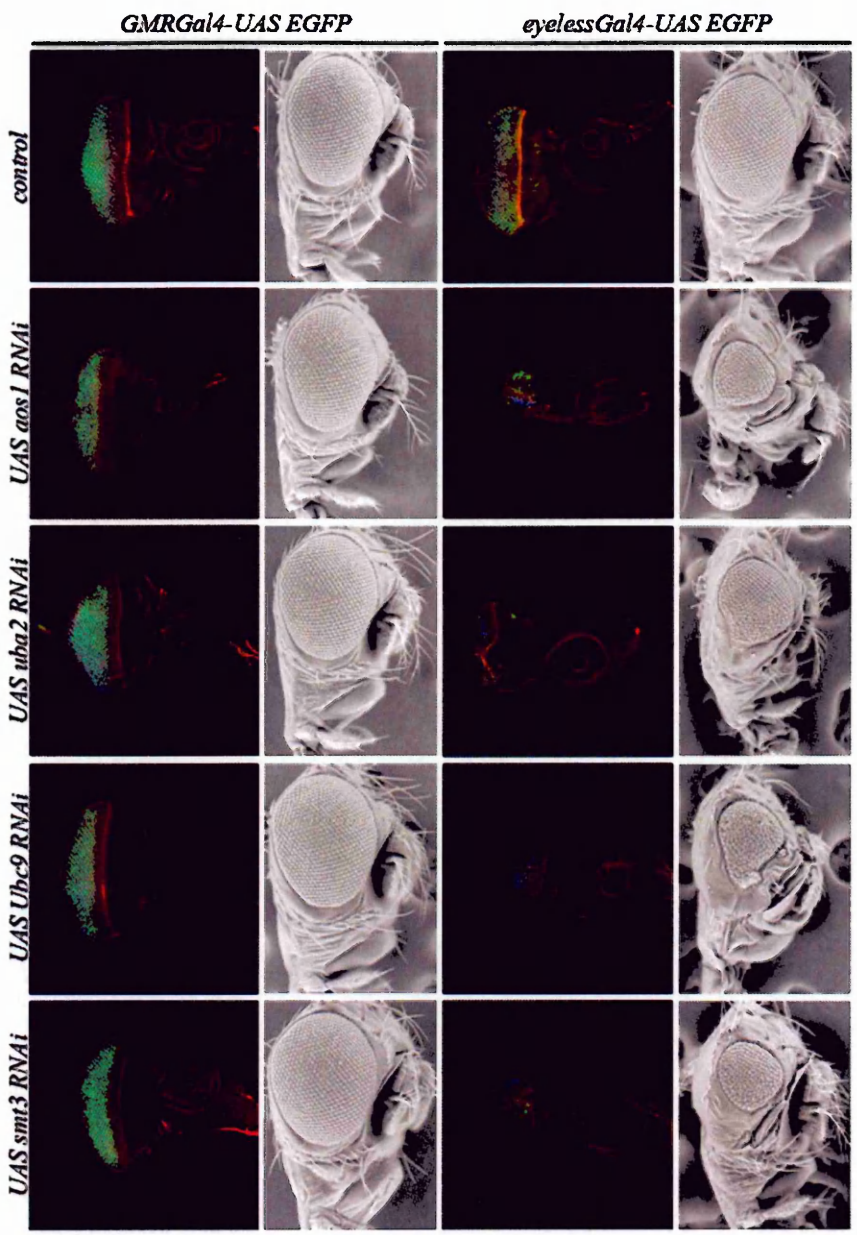

Figure 22

RNAi knock down of the SUMOylation pathway components in the eye imaginal discs. The GMRGal4 driver is expressed in the differentiated cells behind the morphogenetic furrow while the Eyeless Gal4 driver is expressed both in dividing cells in front of the furrow and differentiated cells posterior of the furrow. GFP marks the expression pattern of these Gal4 drivers, as it is shown in control discs (top panels). The discs are stained with phalloidin (red) and anti-elav (blue)

Under the control of GMRGal4 driver the expression of RNAi that targets *aos1*, *uba2*, *ubc9* and *smt3* in the differentiated cells of the eye disc has no effect in the imaginal disc or the formation of the adult eye. In contrast, the same RNAi lines when they are expressed with EyelessGal4 they cause reduction in imaginal disc size which results in small adult eye as well. The phenotypic defects from the RNAi expression vary in severity which ranges from weaker eye size reduction to pupal lethality in cases that the whole head fails to form and the flies never emerge from pupa.

Chapter 4

Cell cycle profile analysis in S2 cells upon *aos1* knock down with dsRNA

aos1 knockdown in S2 cells eliminates Aos1 protein but does not affect cell cycle progression

The analysis of the *aos1* mutant phenotype *in vivo* showed that Aos1 is required for cell cycle progression in imaginal discs, and particularly for the passage through mitosis. In order to test whether Aos1 (and subsequently SUMOylation) is required for cell division in every cell type, the effect of Aos1 depletion on Schneider 2 (S2) cell culture was examined.

The knock down of Aos1 in S2 cells was performed with two different non-overlapping dsRNAs. In this way the phenotypic defects caused from possible off-target genes can be distinguished from the defects which are due to Aos1 elimination and will be common in both dsRNAs. For negative controls, S2 cells were treated with dsRNA against a plasmid vector backbone sequence which does not interfere with any expressed gene in the cells.

The Aos1 protein was gradually eliminated from S2 cells upon dsRNA treatment. On the second day of the knockdown experiment, Aos1 protein levels were already dramatically reduced. By day 4, Aos1 could not be detected (Fig 23A). The cell cycle profile of knocked-down S2 cells was analyzed by FACS at different time points. Surprisingly, there was no significant difference between the cell cycle profile of the Aos1 knockdown cells and the cell cycle profile of the control cells (Fig 23B).

The dsRNA knock-down experiment suggests that cell cycle progression is not affected by Aos1 elimination in S2 cells. However, Western blot analysis showed that global SUMOylation levels were not changed significantly in the knock down cells, although Aos1 was reduced beyond detection (data not shown). Therefore, the unaffected SUMOylation could account for the unaltered cell cycle profile of these cells. One possible explanation is that there is still remaining Aos1 protein in knocked down

cells that cannot be detected with Western blot, but it is sufficient for maintenance of SUMOylation.

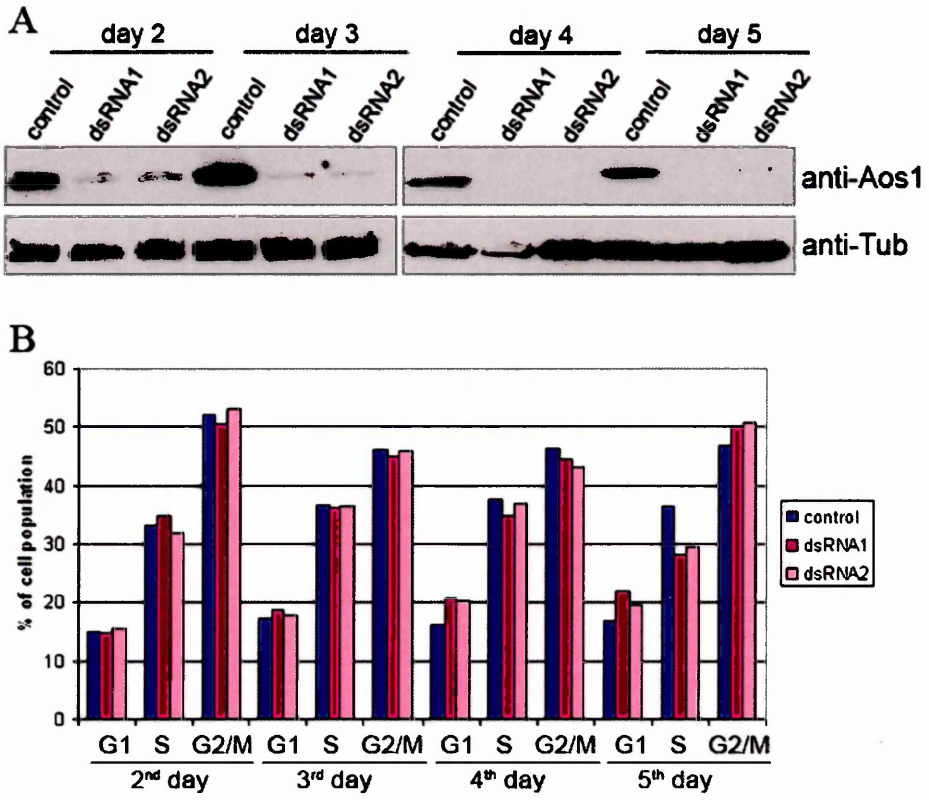

Figure 23
Aos1 knock down in S2 cells
A. western blot analysis showed that *aos1* knock down results in gradual elimination of the Aos1 protein. With both dsRNAs the Aos1 protein is already reduced on day 2 of the experiment compared with the control cells and it is undetectable on day 4 of the dsRNA treatment. Tubulin is used for loading control.
B. FACS analysis of the *aos1* knocked down cells in different time points of the dsRNA treatment showed that, despite the absence of Aos1 protein, there is no significant difference in the cell cycle profile of these cells compared with the control.

smt3 (SUMO) knockdown in S2 cells eliminated SUMO protein and caused cell cycle progression defects in more than one phase

Aos1 knockdown had no effect on cell cycle progression of S2 cells, probably due to unaltered SUMOylation. For this reason, the *smt3* gene was knocked down in order to reduce SUMOylation and observe the effect of this reduction in cell cycle profile of

the knocked down cells.

The *smt3* knock down by dsRNA reduces Smt3 protein and cause a blockage of cell cycle progression at the G1/S transition in S2 cells (Nie et al., 2009). By using the same sequence of *smt3* gene for the dsRNA knock down, we found that the Smt3 protein was significantly reduced after 3 days of dsRNA treatment (Fig 24A). FACS analysis of the *smt3* knockdown cells at different time points showed that the cell populations in G1 and G2/M were increased, while the percentage of cells in S phase was decreased compared with controls (Fig 24B). The Smt3-depleted cells exhibited the same distribution pattern in the different cell cycle phases for all time points that were examined. The defect in cell cycle progression of the *smt3* knocked down cells was also apparent in the cell number, which was reduced compared with the control cells.

In the previous study, the *smt3* knocked down S2 cells are found to accumulate gradually in G1which is consistent with cell cycle arrest in G1/S (Nie et al., 2009). The reproduction of this result was not achieved, as in our hands both G1 and G2/M cell populations were retained while the number of cells entering the S phase was minimal. This suggests that lack of SUMOylation in S2 cells can cause cell cycle blockage in both G1/S transition and passage through mitosis.

In summary, contrasting with imaginal disc cells which require SUMOylation only for the progression through mitosis, it seems S2 cells display an additional requirement of SUMOylation in the G1/S transition. This could reflect a difference in the regulation of cell cycle between cells *in vivo* and in cell culture condition. Nevertheless, a requirement for SUMOylation in cell cycle progression seems to be common in different cell types and *in vivo* and in cell culture systems.

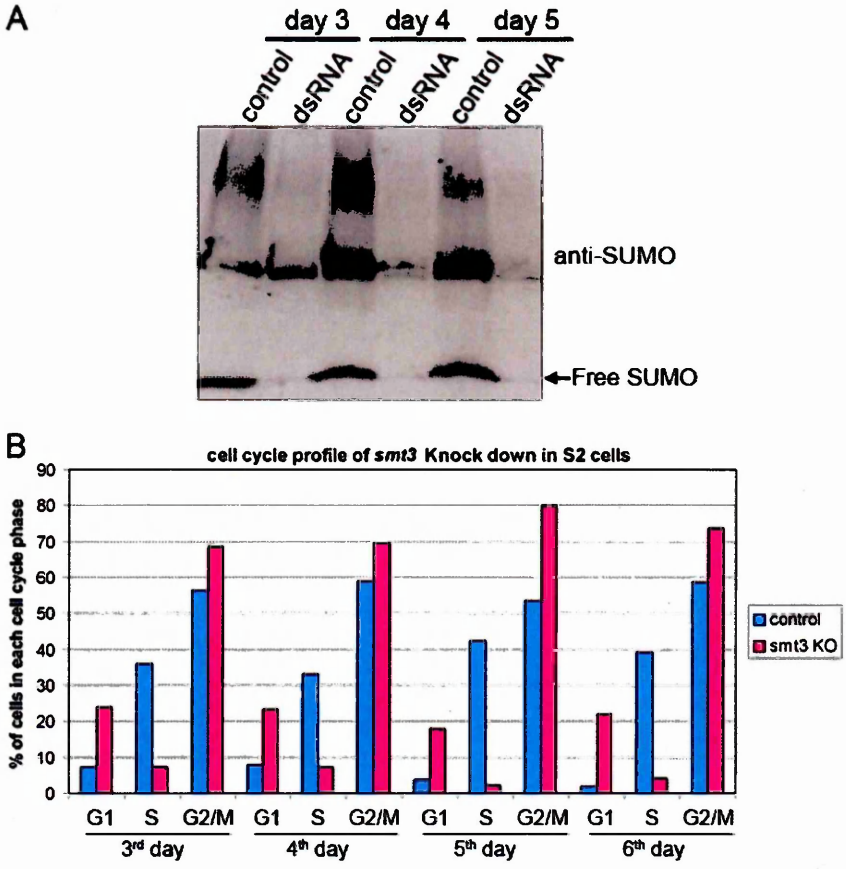

Figure 24

Smt3 knock down in S2 cells

A. Western blot analysis showed that *smt3* knock down upon dsRNA treatment of S2 cells results in reduction of the conjugated and free Smt3 protein from day 3 of the experiment.

B. FACS analysis of the *smt3* knock down cells was used in order to observe the effect on cell cycle progression of the depletion of SUMOylation. In all different time points that were examined the *smt3* knocked down cells exhibit an increase in the percentage of cell population in G1 and G2/M phase compared with the control cells, while the cell population in S phase is almost eliminated. This result suggests that the cell cycle progression in the *smt3* knocked down cells is blocked both in G1/S transition and in the passage through mitosis.

SUMOylation and development

In the present study, the role of the Aos1/Uba2 E1 SUMO activating enzyme in development of *Drosophila* was examined. We show that *aos1* and *uba2* mutant animals exhibit late 3rd instar/early pupal lethality and a severe disruption of imaginal disc and optic lobe development. The $aos1^{Golic1,2}$ null mutants also displayed an inability to form pupae, which is consistent with a proposed requirement for SUMOylation in metamorphosis (Talamillo et al., 2008b).

The relatively late lethality of the E1 enzyme mutant animals can best be explained by a maternal rescue during embryogenesis and by the fact that during larval development cell proliferation is restricted to imaginal cells. It has been shown that there is maternal deposition for all the components of the SUMO conjugation pathway (Hashiyama et al., 2009), and the presence of SUMO conjugation machinery in the egg is required for early cell divisions in the embryo. Removal of maternal Smt3 with induction of germ line clones, leads to embryonic lethality (Nie et al., 2009). We also observed that upon induction of $aos1^{17744}$ germ line clones, the embryos failed to hatch (data not shown).

In early embryos, *aos1* transcript is ubiquitous due to maternal deposition. Later, *aos1* is expressed mainly in the CNS, in the ventral nerve cord, and in the gonads (BDGP expression data). Similarly, *smt3* and *uba2* are expressed primarily in CNS and gonads at late stage embryos (Hashiyama et al., 2009). At the 3rd instar larval stage, we observed that *aos1* is highly expressed in the imaginal discs and the CNS. Thus, *aos1* mutant animals exhibit defects in those tissues in which the *aos1* gene is most highly expressed. It is possible that there were defects in the gonads of the *aos1* mutant larvae as well, but these tissues were not examined closely. The tissues that were most strongly affected by loss of *aos1* (imaginal discs and CNS) consisted of proliferating

cells, and we showed that *aos1* is indeed required for cell proliferation. In contrast, larval tissues comprised of non-diving, endoreplicating cells, were not affected by Aos1 depletion.

Some mutations of *lwr* seem to resemble the phenotype that is produced by *aos1* and *uba2* mutations. The lwr^{13} /lwr^{13} homozygous mutant animals and the trans-heterozygotes lwr^{4-3}/ lwr^{13} and lwr^{5}/ lwr^{13} animals die at the third install larval stage (Xiuli et al., 2003). In contrast, the *semi* class of *lwr* mutants die much earlier, at late embryonic or first instar larval lethality (Epps and Tanda, 1998). It is unclear though, how *semi* mutants and lwr^{13}, which is molecular null, have so different phenotypes.

Animals mutant for smt3 (*Drosophila* SUMO) are reported to die at the early second instar stage (Nie et al., 2009). Thus, mutations for *smt3* cause lethality in earlier developmental stages than the *aos1* and *uba2* mutations. It is not clear what the reason for this difference is, but it could be due to differential protein perdurance. Smt3 maternal protein might decline faster than *aos1* or *uba2*, and thus the embryonic divisions in *smt3* zygotic mutants are disrupted, leading to defects in later developmental stages. However, even in the case that maternal Aos1 and Uba2 decline at the same rate as SUMO, limited Smt3 protein could lead to lower level of SUMOylation than limited *aos1*, as one molecule of the E1 enzyme could catalyze many SUMO conjugation reactions.

SUMOylation and cell cycle progression

We showed that *aos1* mutant imaginal disc cells arrest in G2/M using FACS analysis (Fig 19). The accumulation of SUMOylation-deficient cells in G2/M has been observed both in yeast and in zebrafish upon ubc9 depletion (Nowak and Hammerschmidt, 2006; Seufert et al., 1995). However it is not clear what causes the

cell cycle arrest in G2/M in SUMOylation deficient cells. We examined the possibility that it is caused by DNA damage checkpoint activation, but H2Av loci were not observed in *aos1* mutant cells (data not shown). Next, the activation of Cdk1 as defined by the presence of mitotic Cyclins was tested. Both Cyclin A and Cyclin B were present in *aos1* mutant cells (Fig 21). Similarly, in *ubc9* (E2) mutant yeast cells mitotic Cyclins are stabilized (Ref). The effect of SUMOylation in degradation of mitotic Cyclins could be direct, but so far there is no evidence that Cyclins are modified by SUMO. In contrast, it is reported that Pds1 (securin) is also stabilized in Δsmt3 or Δubc9 mutant yeast cells (Dieckhoff et al., 2004). Pds1 is an APC substrate like the mitotic Cyclins, and the same study shows that more APC substrates are stabilized in SUMOylation deficient cells. Thus, it seems that lack of SUMOylation affects APC mediated proteolysis. However, it is not known if any of the APC subunits are modified by SUMO, or if other factors that regulate APC activity need to be SUMOylated. In addition, we did not observe any metaphase arrest in *aos1* mutant disc cells, which indicates that there must be earlier defects in these cells apart from the inefficient degradation of substrates by the Anaphase Promoting Complex.

The cell cycle arrest in G2/M that is observed in SUMOylation deficient cells indicates that DNA replication can occur in these cells. In zebrafish, it is shown that a small percentage of mutant cells undergo one more round of DNA replication and they have 8n DNA content (Nowak and Hammerschmidt, 2006). In Uba2 depleted yeast cells also it is reported that about 10% of the mutant cells have two buds (Dohmen et al., 1995). Hence, it seems that some mutant cells upon blockage of progression through mitosis can re-enter the cell cycle without the previous completion of mitosis and become polyploid. These observations indicate that the rest of the cell cycle phases (but not mitosis) can be completed in the absence of SUMOylation. We also showed that endoreplication (S-phase progression) is not affected in larval cells of

aos1 mutant animals. These results indicate that in *Drosophila* cell division, SUMOylation is primarily required for G2-M progression.

In contrast, SUMO dsRNA knockdown in S2 cells results in cell cycle arrest at G1/S, before they replicate their DNA (Nie et al., 2009). We were also able to confirm the G1/S progression defect in S2 cells upon SUMO knockdown, with the exception that we observed G2/M progression defect in these cells as well. Surprisingly, this requirement for SUMOylation in the G1/S transition was not observed *in vivo*. One possible explanation is that cell cycle regulation by SUMOylation is different in S2 cells than in imaginal discs. An alternative explanation could be that SUMOylation is required for cell cycle progression through both S phase and mitosis, but there is a different threshold for each of them. S-phase, for example, could require lower levels of SUMOylation than mitosis. Thus, as the amount of Aos1 protein declines in a mutant cell clone, the blockage at G2/M is the first to be met. Following this logic in whole mutant animals, endoreplication of larval cells could be sustained by minimal levels of maternal Aos1 protein while the proliferating imaginal discs would require much higher protein levels in order to divide normally. In this scenario, depletion of SUMO itself in S2 cells probably leads to sudden drop of SUMOylation levels below the threshold requirement for both S phase progression as well as mitosis. Nevertheless, regardless of the specific cell cycle phase requirement, SUMOylation seems to be necessary for the cell cycle progression both *in vivo* and in cell culture.

SUMOylation is required for dividing cells but it is probably dispensable for viability of non-dividing cells

Our results from mosaic analysis in imaginal discs indicate that *aos1* mutant cells exhibit cell cycle progression defects and they die through apoptosis (Fig 15). Similar

observations are reported for the Inner Mass Cells in the mouse embryos that are Ubc9 deficient (Nacerddine et al., 2005). Also, in yeast depletion of Aos1, Uba2 and Ubc9 causes cell cycle arrest and ultimately lysis of the cells (Dohmen et al., 1995; Johnson et al., 1997; Seufert et al., 1995). It is therefore clear that proliferation is affected by disruption of the SUMOylation pathway through ablation of the E1 or E2 enzyme.

Given the broad range of functions for SUMOylation in previous studies, it was unexpected that non-dividing cells were unaffected by the lack of SUMOylation in our experiments. Consistent with our findings, however, trophoblastic cells in *ubc9* mutant mice embryos are viable and morphologically normal (Nacerddine et al., 2005). Further, differentiation is not inhibited in *ubc9* morphant zebrafish cells (Nowak and Hammerschmidt, 2006). In the *Drosophila* eye disc, we showed that GMR-expressing differentiated cells were not dying upon *aos1-, uba2-, ubc9-* and *smt3*-targeting with RNAi (Fig 22), and these discs were able to form adult eyes that were indistinguishable from *wild type*. We obtained similar results when we used the *Eval-Gal4* driver for the expression of *aos1* and *uba2* RNAi in all differentiated neurons of the entire animal (data not shown). In addition, we showed that the endoreplicating larval cells in *aos1* and *uba2* mutant animals were not affected by the loss of SUMOylation.

One possible explanation for the lack of observed phenotypes in non-dividing cells would be that the dividing cells dilute their supply of the enzyme more rapidly, making them more sensitive to loss of pathway components. However, it is shown that proliferating tissues express higher amounts of the SUMO pathway enzymes, which would serve to compensate for the loss of original proteins through dilution and to facilitate cell division. In zebrafish, in situ hybridization showed that *ubc9* is expressed ubiquitously at early embryonic stages but it is primarily expressed in proliferating tissues at later developmental stages (Nowak and Hammerschmidt, 2006). Similarly, in *Drosophila* the expression of *smt3* and *uba2* is higher the CNS and gonads in late em-

bryonic stages (Hashiyama et al., 2009). Expression of *aos1* is also reported to be elevated predominantly in the nervous system and gonads during late embryogenesis (BDGP database). Moreover, we observed using in situ hybridization that at the 3rd instar larval stage *aos1* is expressed mainly in imaginal discs and CNS, which are proliferating tissues during larval development.

These results suggest that there is an increased production of the SUMO conjugation machinery in proliferating cells. This is also demonstrated in the eye disc, where the expression profiles of diving and differentiated cells were analysed by SAGE (Jasper et al., 2002). In this study, *uba2* and *ubc9* (*lwr*) were found to be expressed differentially in the eye disc cell populations. Specifically, *uba2* is expressed about twofold more in proliferating cells than the GMR-expressing differentiated cells, and approximately fourfold higher than in differentiated photoreceptors that express *sevenless*. For *ubc9* (E2), proliferating cells express about 3x higher levels compared with the GMR-expressing non-dividing cells, and ubc9 transcript is almost absent in the *sevenless*-expressing cell population (Jasper et al., 2002).

Thus, it seems that proliferating cells have a higher demand for SUMOylation than the differentiated cells and upon differentiation the production of SUMO pathway component declines. It is also possible that the SUMOylation-deprived cells stop dividing before they dilute the enzymes of SUMO pathway below the level that is found in differentiated cells. In contrast, it seems that the non-dividing cells can tolerate a reduction in SUMOylation. GMR-expressing differentiated cells in the eye disc did not die upon RNAi targeting of the SUMO conjugation machinery and they formed a normal adult eye (Fig 22). The larval tissues of *aos1* and *uba2* mutant animals had severely reduced SUMOylation compared with wild type larval cells, as shown by Western blot analysis (Fig 14). However, these cells could still endoreplicate and they sustained organismal survival and normal cellular homeostasis through a prolonged

larval period, which in the case of *aos1*^{Golic1,2} mutant animals lasted for more than ten days (Fig 10C).

Taken together, our results combined with previous observations in *Drosophila* and other organisms suggest that SUMOylation is required for cell division and subsequently for survival in proliferating cells. Our studies also indicate that SUMOylation is probably not essential in non-mitotic cells, unless minimal SUMO conjugation is sufficient for all cellular functions. SUMOylation therefore is essential for organism viability, as without it proliferation is blocked and development cannot be completed, while for cell viability SUMOylation might be dispensable.

If SUMOylation is not absolutely required for cell viability or homeostasis, an obvious question is why non-dividing larval cells still express SUMO pathway components and retain SUMO-conjugation, as shown by Western blots (Fig 14). One possibility is that the presence of SUMOylation is important for the robustness of some processes in these cells. It is also possible that SUMOylation provides a safety mechanism for the cells upon environmental challenges. It has been shown that various stresses increase SUMOylation levels (Bossis and Melchior, 2006) and that viral proteins can interfere with SUMO conjugation machinery (Chiocca, 2007). Thus, retaining low levels of SUMOylation could facilitate the rapid response of cells to changes in the conditions of environment or protect the cells against infections. However, further studies are required in order to understand the role of SUMOylation in cells under different environmental conditions.

Finally, the possibility that SUMOylation is required primarily in dividing cells could provide a new approach in the treatment of pathological conditions that are caused by uncontrolled cell proliferation, like cancer. Targeting and reducing SUMOylation could prevent cell proliferation of malignant cells leaving unaffected the "healthy", differentiated cells. This would be advantageous in comparison with drugs

treatments that they generate severe side effects, for example, drugs that inhibit proliferation through spindle formation and can also affect the structure of microtubules in other cells, such as neurons.

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